The Role of WNT5A
in the Pathogenesis of Aggressive Fibromatosis

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Summary

Fibromatoses belong to the soft tissue tumors and arise in the connective tissue. They represent semimalignant lesions, characterized by invasive growth and a tendency to local recurrence, but lacking the formation of metastases. Depending on the sites of involvement, they are categorized as deep and superficial fibromatoses. Deep or aggressive fibromatoses occur extra- and intraabdominally, whereas the predilection sites for superficial fibromatoses are the palmar and plantar aponeuroses. The tumor cells exhibit features of myofibroblasts, a cell type that possesses attributes of both fibroblasts and smooth muscle cells. They are embedded in an abundant, collagen rich extracellular matrix. Aggressive and superficial fibromatoses are characterized by a nuclear accumulation of N-terminally dephosphorylated, active β-catenin. β-catenin exerts a crucial function in the adhesive complex at the cell membrane, as well as in the nucleus as a transcription cofactor of the canonical Wnt pathway. The nuclear accumulation of β-catenin is in most of the cases of aggressive fibromatoses derived from a biallelic mutation of the tumor suppressor gene adenomatous polyposis coli (APC; familial adenomatous polyposis) or a monoallelic mutation of the oncogene β-catenin itself (sporadic form). The reason for the nuclear accumulation of β-catenin in superficial fibromatosis remains to be elucidated.

The present study is divided into three chapters. The aim of the first chapter was the identification of genes differentially expressed in aggressive and superficial fibromatoses as compared to reference normal fibrous tissue. This was accomplished by means of Agilent gene expression microarrays. In addition, the differentially expressed genes between the tumors were analyzed. Newly identified regulated genes may serve as markers for differentiating those soft tissue tumors, as well as contribute to the understanding of the common and discriminative mechanisms being responsible for the pathogenesis of aggressive and superficial fibromatoses. Chapter II covers the description of experiments aimed at the establishment and characterization of primary tumor cell and normal fibroblast cultures. They should represent the basis for studying the functional relevance of proteins encoded by the determined differentially expressed genes. Chapter III deals with the impact of the Wnt ligand WNT5A on the canonical and non-canonical Wnt signalling pathways in the primary tumor cell culture of an aggressive fibromatosis (Aggr6). WNT5A was found to be regularly highly overexpressed in tumor tissues of aggressive fibromatosis as compared to superficial fibromatosis or normal fibrous tissue.

Chapter I

For the Agilent 60mer-oligonucleotide microarray experiments, total RNA from 10 individual aggressive fibromatoses and 15 individual superficial fibromatoses were pooled to generate an aggressive fibromatosis RNA pool and a superficial fibromatosis RNA pool. As a reference, RNA samples from five fibrous tissues were pooled. In addition, RNA samples derived from 5 single aggressive fibromatoses and 1 single superficial fibromatosis were hybridized on individual arrays. To find genes that most significantly differentiate in their expressions the tissues analyzed, the following stringent selection criteria were applied: an absolute expression level of at least 500, a fold-change of at least two and a p-value of 0.01 or less. By this means, 2’429 known genes could be identified that differentiate the three tissue pools from

each other (aggressive fibromatosis / reference fibrous tissue: 1'113 overexpressed (↑), 860 downregulated (↓); superficial fibromatosis / reference fibrous tissue: 724 ↑, 707 ↓; superficial fibromatosis / aggressive fibromatosis: 226 ↑, 275 ↓). A hierarchical clustering analysis, based on the ratios of the absolute expression levels of those genes between the tumor tissues and the reference fibrous tissue, built two clusters, separating the samples of aggressive fibromatosis from the ones of superficial fibromatosis. The differential expressions of 30 selected genes could be confirmed without exception using real-time RT-PCR. One of those genes represents WNT5A, which is regularly highly overexpressed in aggressive fibromatosis as compared to superficial fibromatosis and reference fibrous tissue.

Using the software 'DAVID', about half of the selected 2'429 genes could be associated with a biological process.

In the list of genes commonly differentiating the two tumors from the reference fibrous tissue, the following biological processes were statistically significantly overrepresented: Wnt signalling pathway, TGFβ signalling pathway, PI3K-AKT signalling pathway, extracellular matrix (ECM) components, ECM-receptor interaction, adherens junction and cell adhesion molecules (CAMs), proliferation, cytoskeleton, as well as complement and coagulation cascades. Whereas the Wnt signalling pathway is known to play a crucial role in the pathogenesis of both aggressive and superficial fibromatoses, the formation of superficial fibromatosis has been associated with a deregulated TGFβ signalling pathway. The involvement of the PI3K-AKT pathway in the pathogenesis of fibromatoses has been described so far in only one report in superficial fibromatosis. Both tumors are characterized by a pronounced accumulation of ECM proteins. Since tumor cells of fibromatoses represent myofibroblasts, differences in the composition of the cytoskeleton, especially of the alpha smooth muscle actin (α-SMA) containing 5nm actin filaments, are obvious. Alterations in cell-cell and cell-ECM interactions, as well as dysfunctions in the regulation of proliferation, are commonly involved in tumorigenesis.

Interestingly, in the list of genes differentiating the two tumors from each other, most of the above mentioned biological processes are statistically overrepresented as well. The exceptions represent the PI3K-AKT pathway and the process of proliferation that commonly differentiate the two tumors from the reference fibrous tissue, but do not discriminate aggressive from superficial fibromatosis. Those differentially expressed genes represent attractive candidates for distinguishing the two tumors on the protein level using immunohistochemistry. Examples are thrombospondin 4 (THBS4), an ECM component that positively influences the activity of the TGFβ signalling pathway and was found to be highly overexpressed in superficial fibromatosis, Amphiregulin (AREG), a member of the epidermal growth factor (EGF) family, and WNT5A (a ligand of the Wnt signalling cascades). The latter two are highly overexpressed in aggressive fibromatosis.

The identities of the differentially expressed components of the three signalling pathways were analyzed in more detail. They were grouped according to their functions into pathway activity inducers, inhibitors and pathway target genes. This approach aimed to deduce informations about differential activities within these pathways between the tissues analyzed. Although some restrictions of the validity of the resulting informations must be considered, the following findings could be deduced from the analysis of the data:

An overexpression of pathway activators and target genes indicates that the Wnt and the TGFβ signalling pathway are more active in both tumors as compared to the reference fibrous tissue. In addition, an upregulation of Wnt pathway activity inhibitors in fibromatoses suggests that a negative feedback loop mechanism may try to counterbalance an excessive Wnt signalling pathway activity in both tumors. In the Wnt and the TGFβ pathway, there is an extensive overlap of differentially expressed components in aggressive and superficial fibromatoses, reflecting prominent similarities in the deregulation of those pathways in both fibromatoses.

For the analysis of the PI3K-AKT pathway, a different approach was applied. The PI3K-AKT pathway exerts diverse effects on cells, including an inhibition of apoptosis and an activation of proliferation. Thereby, the serine threonine kinase AKT plays a central role, by modulating the function of diverse downstream proteins, both on the protein (phosphorylation, ubiquitination) and the RNA level (transcription).

The analysis of pathway inducers and inhibitors upstream of AKT did not reveal a differential activity of the PI3K-AKT pathway between tumor tissues and reference fibrous tissue. Downstream of AKT, several components of this pathway were found to be differentially expressed between fibromatoses and normal fibrous tissue. Whereas the overexpression of cyclin D1 (CCND1) and hypoxia-inducible factor 1 α subunit (HIF1A), and the downregulation of cyclin / cyclin-dependent kinase complex inhibitor 1B (CDKN1B) can be explained by an activated PI3K-AKT signalling pathway, the reason for the transcriptional regulation of the other pathway components is unknown. However, the net effect of those differentially expressed components is an induction of proliferation and an inhibition of apoptosis.

In a recent paper, the authors proved an enhanced activity of the PI3K-AKT pathway in superficial fibromatosis as compared to reference fibrous tissue, by demonstrating an increased phosphorylation-dependent activation of AKT (Western blot). This may lead to an additional amplification of the proliferation inducing and apoptosis repressing effects through phosphorylation of the differentially expressed genes found in this study.

In the literature, 243 genes are known to be differentially expressed between fibromatoses and normal fibrous tissue, whereas only a few of them differentiate the two tumors from each other. Most of them were detected by means of gene expression microarray experiments. Therefore, the bulk of genes found to be transcriptionally regulated in this study are unknown up to date. The functional annotation of the regulated genes published in the literature revealed the same biological processes as the ones determined by the analysis of the differentially expressed genes obtained in this study. The published statements for 93 of those genes could be confirmed by our own data, a consensus that is much higher than expected when comparing two randomly selected lists of differentially expressed genes.

Chapter II

For the establishment of primary tumor cell cultures, 7 aggressive fibromatoses (one familial adenomatous polyposis (FAP)-associated form carrying a monoallelic

mutation in APC, 6 sporadic forms, 5 of them characterized by a monoallelic β-catenin mutation) and 5 superficial fibromatoses (without any known mutation) were taken into culture as small tissue pieces or single cell suspensions after a collagenase pre-treatment. Cell cultures of normal fibroblasts from the corium of normal dermal tissues were established in the same way. All cell cultures were characterized by the outgrowth of fibroblast-like cells (aggressive fibromatosis: Aggr1-7, superficial fibromatosis: Super1-5, normal adult dermal fibroblasts: HDF15, 21 and NHDF).

The cell culture derived from the FAP-associated aggressive fibromatosis (Aggr1) carried the same monoallelic APC-mutation as the primary tumor tissue. But since in this tissue, an unknown epigenetic alteration is responsible for the inactivation of the second APC allele, the tumoral origin of the primary cell culture Aggr1 can not be proven. The same accounts for Aggr7 and Super1-5, because the original tumor tissues do not carry any known mutations which could be used as markers. Since in Aggr2-5, the monoallelic β-catenin mutations present in the original tumor tissues are missing, those cells represent normal fibroblasts. On the other hand, the β-catenin point mutation at codon 45 (serine → phenylalanine) in the tumor tissue could also be found in the corresponding primary cell culture Aggr6, undoubtedly proving its tumoral origin. After 23 passages in vitro, this mutation was still present in Aggr6 cells. However, the cells ceased proliferation (senescence); those cells therefore represent a primary cell culture, not an indefinitely proliferating, immortalized cell line.

The endogenous expression of WNT5A was measured in the primary cell cultures Aggr2 and Aggr4 (normal fibroblasts), Aggr7 (unknown) and Aggr6 (tumor cells) by means of gene expression micoarrays (Agilent), and compared with the expressions in the original tumor tissues. There was no correlation between the WNT5A expression in vitro and in vivo. The determination of the WNT5A protein content in the cytoplasm and the cell culture supernatant (Western blot, IP), although proving a correlation with the gene expression, did not reveal the highest values for the tumor cell culture Aggr6. The reason for the overexpression of WNT5A in tumor tissues as compared to normal fibrous tissues and for the variable expression and secretion of WNT5A in vitro, independent of the identity of the culture as a tumor cell or normal fibroblast cell culture, is unknown.

Therefore, with Aggr6, an undoubtedly proven primary tumor cell culture is available for the functional studies described in Chapter III.

Chapter III

Among the abundant possible candidates for subsequent functional studies in cell cultures, the Wnt signalling pathway ligand WNT5A was selected based on the following reasons: 1) WNT5A is highly overexpressed in aggressive fibromatosis as compared to superficial fibromatosis or reference fibrous tissue. This could be observed both by the analysis of pooled RNA samples, as well as by the measurements in individual tumor tissues. 2) WNT5A is a well-known activator of both the canonical Wnt as well as the non-canonical Wnt signalling pathways. The latter include the Wnt-planar cell polarity (PCP), the Wnt-Ca^{2+}, the protein kinase A (PKA) and the signal transducer and activator of transcription (STAT) signalling pathways. 3) WNT5A has been associated both with tumor promoting and tumor

suppressing properties, depending on the type of tumor analyzed. Its role in the pathogenesis of fibromatoses has not been investigated, yet.

The activity of the canonical Wnt signalling pathway was measured using a TCF-reporter gene assay (Luciferase assay) and the quantification of the active, dephosphorylated form of β-catenin (Western blot). The activity of the PKA pathway was determined by the analysis of the phosphorylated, active form of the transcription factor CREB (Western blot). To measure the activity of the Wnt-PCP, the Wnt-Ca^{2+} and the STAT pathway, transcription factor assays were used.

In agreement with the classical theory whereupon a monoallelic S45F β-catenin mutation leads to the accumulation of dephosphorylated, active β-catenin, the baseline activity of Wnt signalling pathway is in Aggr6 tumor cells higher than in cell cultures of normal fibroblasts. Whereas endogenously produced WNT5A does not exert any effect on the activity of the canonical Wnt pathway, stimulation with recombinant WNT5A led to a pronounced increase of its signalling activity in the tumor cell culture Aggr6, but not in the cell cultures of normal fibroblasts. Treatment with recombinant WNT5A led also to a selective stimulation of the activities of the non-canonical Wnt pathways Wnt-PCP and PKA in Aggr6 cells. By means of specific activators and inhibitors of those two pathways, a crosstalk with the canonical Wnt pathway could be excluded.

A cell proliferation assay revealed that recombinant WNT5A selectively increased the proliferation of the tumor cell culture Aggr6, but did not alter the proliferation rate of normal fibroblasts. Whereas the inhibition of the canonical Wnt pathway and the Wnt-PCP pathway by the use of specific pathway inhibitors did not impair WNT5A's positive effect on cellular proliferation, the abrogation of the PKA pathway almost completely abolished the proliferative activity of Aggr6 cells. Thus, of the three pathways known to be activated by WNT5A, the PKA pathway elucidated to be the only one decisively involved in the regulation of proliferation of Aggr6 tumor cells, both in terms of their basal proliferation rate and the WNT5A-dependent stimulation of proliferation.

A type I collagen Boyden chamber invasion assay was used to measure the invasive growth behaviour of the individual cell cultures. Aggr6 tumor cells behaved similarly as cell cultures of normal fibroblasts. The addition of recombinant WNT5A did not alter the invasive growth behaviour, neither of Aggr6 tumor cells, nor of normal fibroblasts.

The numbers of differentially expressed genes obtained by a gene expression microarray experiment impressively reflected the above findings concerning a selective responsiveness of the tumor cell culture Aggr6 towards recombinant WNT5A: it exerted a pronounced effect on the gene expression in Aggr6 cells, but hardly affected transcription in normal fibroblasts.

In search of reasons for the differential impact of recombinant WNT5A on Aggr6 tumor cells and normal fibroblasts, respectively, microarray gene expression data were analyzed to detect differentially expressed WNT5A-binding Frizzled (Fzd) receptors. It could be shown that Aggr6 tumor cells overexpress Fzd1, Fzd2 and Fzd7 as compared to normal fibroblasts. Those Fzd receptors are accordingly regulated also in aggressive fibromatosis tissues in comparison to reference fibrous

tissue. This led to the suspicion, that Fzd1, Fzd2 and Fzd7 receptors are responsible for WNT5A binding on tumor cells. However, downregulation of all three receptors by means of siRNAs transfected into Aggr6 cells did not lead to a reduced responsiveness of these cells towards recombinant WNT5A.

Another explanation would be that the enhanced basal canonical Wnt pathway activity in Aggr6 tumor cells induces the expression of WNT5A receptors. Although an overexpression of β-catenin mutated at codon 45 (Δ45-β-catenin) in normal fibroblasts led to an increased canonical Wnt pathway activity, it did not allow a further stimuation of this pathway by recombinant WNT5A. Therefore, the mechanisms being responsible for the observed discrepancy between Aggr6 cells and the cell cultures of normal fibroblasts in the responsiveness towards WNT5A remain to be elucidated.

In summary, the obtained in vitro results provide the basis for the understanding of the mechanisms, by which WNT5A, highly overexpressed in aggressive fibromatosis, exerts its proliferation inducing effect.

Zusammenfassung

Fibromatosen gehören zu den Weichteiltumoren und entstehen im Bindegewebe. Als semimaligne Tumoren sind sie charakterisiert durch ein invasives Wachstum und durch die Tendenz zur Bildung von Lokalrezidiven. Metastasen entstehen keine. Aggressive Fibromatosen werden wegen ihrer Lokalisation auch als tiefe Fibromatosen bezeichnet und den superfiziellen Fibromatosen (Palmar- und Plantaraponeurosen) gegenübergestellt. Die Tumorzellen gleichen Myofibroblasten, einem Zelltyp mit Merkmalen sowohl von Fibroblasten als auch von glatten Muskelzellen. Sie sind eingebettet in eine abundante, kollagenreiche extrazelluläre Matrix. Gemeinsam ist den Fibromatosen eine nukleäre Akkumulation der aktiven, dephosphorylierten Form des β-catenins. Dieses spielt neben seiner zentralen Funktion im Zelladhäsionskomplex eine wichtige aktivierende Rolle im Wnt-Signaltransduktionsweg als Transkriptions-Cofaktor. Die Akkumulation von β-catenin ist in aggressiven Fibromatosen in den meisten Fällen auf eine biallelische Mutation des adenomatous polyposis coli (APC) Tumorsuppressor-Gens (familiäre adenomatöse Polyposis Coli) oder eine monoallelische Mutation des Onkogens β-catenin selber (sporadische Formen) zurückzuführen. Der Grund für die Anreicherung des β-catenins im Nukleus der superfiziellen Fibromatosen ist unbekannt.

Die vorliegende Arbeit umfasst drei Kapitel. Ziel des ersten Kapitels war es, mittels der Mikroarray-Genexpressions-Technologie (Agilent) Gene zu identifizieren, die im Tumorgewebe der aggressiven und superfiziellen Fibromatose im Vergleich zu normalem fibroblastärem Bindegewebe differenziell exprimiert sind. Ausserdem wurden die Genexpressions-Unterschiede zwischen den beiden Tumoren untersucht. Neu entdeckte differentiell exprimierte Gene können einerseits als Marker für die Unterscheidung dieser Weichteiltumoren und anderseits zum Verständnis der für die Pathogenese dieser beiden Tumoren verantwortlichen gemeinsamen und unterschiedlichen Mechanismen beitragen. Durch die im Kapitel II beschriebene Etablierung und Charakterisierung von Primärkulturen von Tumorzellen und normalen Fibroblasten sollte die Möglichkeit geschaffen werden, die funktionelle Bedeutung differentiell exprimierter Genprodukte zu studieren. Kapitel III befasst sich mit der Wirkung des Wnt Liganden WNT5A auf den kanonischen und die nicht-kanonischen Wnt-Signaltransduktionswege in Tumorzellen der Primärkultur einer aggressiven Fibromatose (Aggr6). WNT5A wird im Tumorgewebe aggressiver Fibromatoses regelmässig überexprimiert.

Kapitel I

Für die Genexpressionsanalyse auf Agilent-Oligonukleotid-Mikroarrays wurde die RNS aus dem Tumorgewebe von je 10 aggressiven und 15 superfiziellen Fibromatosen sowie aus 5 Exzisaten von normalem Bindegewebe extrahiert und für die Herstellung des Genexpressionsprofils gepoolt. Zusätzlich wurden 5 einzelne aggressive Fibromatosen und eine weitere superfizielle Fibromatose analysiert. Um Gene zu finden, die in ihrer Expression die untersuchten Gewebe am signifikantesten voneinander unterscheiden, wurden folgende stringenten Selektionskriterien angewendet: absolute Expressionsstärke ≥ 500, Expressionsverhältnis ≥ 2 oder ≤ 0.5, p-value ≤ 0.01. Auf diese Weise konnten 2'429 bekannte Gene gefunden werden, die zwischen den einzelnen Gewebepools differenziell exprimiert sind: (aggressive

Fibromatose / Bindegewebe: 1'113 überexprimiert (↑), 860 herunterreguliert (↓); superfizielle Fibromatose / Bindegewebe: 724 ↑, 707 ↓ ; superfizielle Fibromatose / aggressive Fibromatose: 226 ↑, 275 ↓). In einer Cluster-Analyse, basierend auf dem Verhältnis der Expressionsstärke dieser Gene im Tumorgewebe, verglichen zum normalen Bindegewebe, ergab sich eine klare Abgrenzung der aggressiven von der superfiziellen Fibromatose. Die differentielle Expression von 30 ausgewählten Genen konnte mittels real-time RT-PCR ausnahmslos bestätigt werden. Eines dieser Gene ist WNT5A, welches in der aggressiven Fibromatose im Vergleich zur superfiziellen Fibromatose und zum normalen Bindegewebe ausnahmslos stark überexprimiert ist.

Mit dem Programm ‚DAVID' konnte etwa die Hälfte der 2'429 selektierten Gene einem biologischen Prozess zugeordnet werden.

Betrachtet man nur die Gene, die die beiden Tumoren gemeinsam vom Bindegewebe unterscheiden, so sind folgende biologischen Prozesse statistisch signifikant übervertreten: Wnt-Signaltransduktionsweg, TGFβ-Signaltransduktionsweg, PI3K-AKT-Signaltransduktionsweg, extrazelluläre Matrix- (ECM)-Komponenten, ECM-Rezeptor-Interaktion, Zelladhäsionskomplex- und Zelladhäsionsmoleküle, Proliferation, Zytoskelett sowie Komplement- und Blutgerinnungs-Kaskaden. Während der Wnt-Signaltransduktionsweg in der Pathogenese sowohl der aggressiven, wie auch der superfiziellen Fibromatose bekanntermassen eine Rolle spielt, wird der TGFβ-Signaltransduktionsweg in der superfiziellen Fibromatose als ursächlich beschrieben. Nur wenig ist bekannt über die Bedeutung des PI3K-AKT-Signaltransduktionsweges für die Entstehung dieser Tumoren. Eine Akkumulierung von ECM-Komponenten ist charakteristisch für beide Fibromatosen. Da es sich bei den Tumorzellen um Myofibroblasten handelt, sind auch die Unterschiede in der Zusammensetzung des Zytoskeletts, namentlich der 5nm Aktin-Filamente (welche das α-SMA glatter Muskelzellen enthalten), verständlich. Veränderte Zell-Zell- und Zell-ECM-Interaktionen, wie auch die unterschiedliche Regulation der Proliferation, sind allgemein bei der Entstehung von Tumoren von Bedeutung.

Von den Genen, die zwischen der aggressiven und der superfiziellen Fibromatose differentiell exprimiert sind, gehören interessanterweise die meisten zu den gleichen signifikant übervertretenen biologischen Prozessen. Ausnahmen sind der PI3K-AKT-Signaltransduktionsweg und die Proliferation, die beide Tumoren in gleicher Weise vom Bindegewebe unterscheiden, nicht aber die beiden Tumoren untereinander. Solche differentiell exprimierten Gene stellen auf Proteinebene attraktive Kandidaten zur immunhistochemischen Unterscheidung der beiden Tumoren dar. Als Beispiele können Thrombospondin 4 (THBS4), Amphiregulin (AREG) und WNT5A genannt werden. THBS4 ist eine ECM Komponente, die einen aktivierenden Einfluss auf den TGFβ-Signaltransduktionsweg ausübt. Dieses Gen ist stark überexprimiert in der superfiziellen Fibromatose. AREG (gehört zur Familie der epidermal growth factors (EGF)) und WNT5A (Ligand der Wnt-Signaltransduktionskaskaden) sind in aggressiven Fibromatosen stark überexprimiert.

Die Identität der differentiell exprimierten Komponenten der drei Signaltransduktionswege wurde genauer analysiert. Dazu wurden sie bezüglich ihrer Funktion in Aktivatoren, Inhibitoren und Zielgene des Signaltransduktionsweges gruppiert mit dem Zweck, Aussagen über die differentielle Aktivität dieser Signalwege in den analysierten Geweben machen zu können. Obwohl bei diesem Vorgehen einige

Einschränkungen bezüglich der Aussagekraft der erhaltenen Resultate gemacht werden müssen, liess sich aus der Analyse der Daten folgendes ableiten:

Die Überexpression von Signalweg-Aktivatoren und Zielgenen deutet darauf hin, dass der Wnt- und der TGFβ-Signaltransduktionsweg in beiden Tumoren aktiver ist als im normalen Bindegewebe. Ausserdem lässt eine verstärkte Expression von Wnt-Signalweg-Inhibitoren in beiden Tumoren darauf schliessen, dass diese in einem negativen Regelkreis dieser Aktivität entgegenwirken. Zwischen den Tumoren besteht eine grosse Übereinstimmung bezüglich der differentiell exprimierten Komponenten in beiden Signaltransduktionswegen.

Eine etwas andere Vorgehensweise wurde für die Analyse des PI3K-AKT-Signaltransduktionswegs angewendet. Die PI3K-AKT Signalkaskade übt unter anderem einen induzierenden Effekt auf die Proliferation und einen inhibierenden Einfluss auf die Apoptose aus, indem sie via AKT (eine Serin/Threonin-Kinase) verschiedenste Proteine in ihrer Funktion durch Phosphorylierung und Ubiquitinierung beeinflusst. Andere Komponenten des Signalweges werden aber auf Stufe Transkription reguliert.

Eine Analyse der Signaltransduktionsweg-Aktivatoren und -Inhibitoren oberhalb von AKT liess auf keine differentielle Aktivität des PI3K-AKT-Signalwegs zwischen den Tumorgeweben und normalem Bindegewebe schliessen. Unterhalb von AKT fanden sich in der vorliegenden Studie mehrere Komponenten, die zwischen den Tumoren und dem Referenzgewebe differentiell exprimiert sind. Die Überexpression von Cyclin D1 (CCND1) sowie von hypoxia-inducible factor 1α subunit (HIF1A) und die Herunterregulierung von cyclin/cyclin-dependent kinase complex inhibitor 1B (CDKN1B) können durch einen aktivierten PI3K-AKT-Signaltransduktionsweg erklärt werden, während die Ursache für die differentielle Expression der anderen Komponenten unbekannt ist. Der Nettoeffekt dieser differentiell exprimierten Komponenten ist eine Induktion der Proliferation und eine Hemmung der Apoptose.

In einer neueren Publikation wird durch den Nachweis von Phospho-AKT die Aktivierung dieses Signalweges in der superfiziellen Fibromatose bewiesen. Möglicherweise führt dies zu einer weiteren Verstärkung des proliferationsfördernden und apoptosehemmenden Effekts der differentiell exprimierten Gene unterhalb AKT.

Aus der Literatur sind 243 Gene bekannt, die zwischen Fibromatosen und normalem Bindegewebe differenziell exprimiert sind. Nur sehr wenige dieser Gene unterscheiden zwischen aggressiver und superfizieller Fibromatose. Die meisten wurden mittels Mikroarray-Genexpressionsstudien entdeckt. Demzufolge war der Grossteil der in dieser Studie identifizierten differentiell exprimierten Gene bisher noch nicht bekannt. Die differentielle Expression von 93 der 243 bekannten Gene konnte bestätigt werden. Diese Übereinstimmung ist statistisch gesehen um einiges grösser als zu erwarten wäre, wenn man zwei beliebige Genlisten miteinander vergleicht. Eine Gruppierung der in der Literatur beschriebenen Gene nach biologischen Prozessen ergibt ein ähnliches Bild, wie es auch in der vorliegenden Studie erhalten wurde.

Kapitel II

Zur Etablierung einer Tumorzellinie wurden 7 aggressive Fibromatosen (eine familiäre Form mit monoallelischer APC-Mutation, 6 sporadische Formen, davon 5 mit und eine ohne β-catenin-Mutation) und 5 superfizielle Fibromatosen (ohne bekannte Mutation) als Gewebestückchen oder als Kollagenase isolierte Zellen in Kultur genommen. Normale Fibroblastenkulturen wurden auf gleiche Weise aus Corium mehrerer normaler Hautexzisate angelegt. In allen Fällen kam es zum Wachstum von fibroblastenähnlichen Primärkulturen (aggressive Fibromatosen: Aggr1-7, superfizielle Fibromatosen: Super1-5, normale adulte dermale Fibrobasten: HDF15, 21 und NHDF).

Die aus der familiären Form der aggressiven Fibromatose ausgewachsenen Zellen (Aggr1) wiesen die gleiche monoallelische APC-Mutation auf wie der Ausgangstumor. Da aber schon in diesem eine nicht identifizierte epigenetische Veränderung für den Ausfall des zweiten Allels verantwortlich ist, kann nicht entschieden werden, ob es sich um eine Tumorzelle oder einen normalen Fibroblasten handelt. Das gleiche gilt für Aggr7 und Super1-5, da die Originaltumoren keine bekannten, als Marker verwendbare Mutationen aufweisen. Da Aggr2-5 die monoallelische β-catenin-Mutationen der Ausgangstumoren nicht zeigen, handelt es sich bei diesen um normale Fibroblasten. Nur Aggr6 konnte durch die Präsenz der gleichen β-catenin-Punktmutation im Codon 45 (Serin→Phenylalanin), die auch schon der Ausgangstumor trägt, eindeutig als Tumorzelle identifiziert werden. Nach 23 Generationen findet sich diese Mutation in den Tumorzellen immer noch. Hingegen kommt es zu einem Wachstumsstillstand (Seneszenz); es handelt sich also um eine Primärkultur und keine permanent wachsende, immortalisierte Zellinie.

Die endogene WNT5A-Expression wurde in den Zellkulturen Aggr2 und Aggr4 (Fibroblasten), Aggr7 (unklar) und Aggr6 (Tumorzellen) mittels der Microarray-Technologie (Agilent) gemessen und mit der Expression im Originalgewebe verglichen. Es konnte keine Korrelation der Expressionsstärke von WNT5A in vitro mit derjenigen in vivo gefunden werden. Die Bestimmung des WNT5A-Proteingehalts im Zytoplasma und im Zellkultur-Überstand (Western Blot, IP) zeigte zwar eine Übereinstimmung mit der gemessenen Gen-Expression, war aber in Aggr6 nicht am höchsten. Der Grund für die WNT5A-Überexpression im Tumorgewebe und für die unterschiedliche Expression und Produktion von WNT5A in Zellkulturen (unabhängig davon, ob es sich um Tumorzellen oder normale Fibroblasten handelt), ist unbekannt.

Somit steht für funktionelle Studien mit Aggr6 bewiesenermassen eine primäre Tumorzellkultur zur Verfügung.

Kapitel III

Aus der grossen Fülle möglicher Kandidaten wurde für die anschliessenden funktionellen Studien in Zellkulturen der Wnt-Signalisationskaskade-Ligand WNT5A aus den folgenden Gründen ausgewählt: 1) Die Expression von WNT5A ist in aggressiven Fibromatosen gegenüber superfiziellen Fibromatosen und normalem Bindegewebe massiv erhöht. Dies gilt sowohl für die Bestimmung aus gepoolter RNS von mehreren Tumoren und Bindegeweben, als auch für die Messung von Einzel-

geweben. 2) WNT5A ist ein bekannter Aktivator sowohl des kanonischen als auch der nicht-kanonischen Wnt-Signaltransduktionswege. Zu den letzteren gehören der Wnt-planar cell polarity (PCP)-, der Wnt-Ca^{2+}-, der Protein Kinase A (PKA)- und der signal transducer and activator of transcription (STAT)-Signaltransduktionsweg. 3) WNT5A wurden in der Literatur sowohl Tumor fördernde als auch Tumor supprimierende Eigenschaften zugeschrieben, je nach Typ des untersuchten Tumors. Im Zusammenhang mit Fibromatosen wurde WNT5A bisher noch nicht untersucht.

Die Aktivität des kanonischen Wnt-Signalweges wurde mittels des TCF-Reportergen-Assays (Luciferase Assay) und der Quantifizierung der aktiven, dephosphorylierten Form des β-catenins bestimmt (Western Blot). Die Aktivität des PKA-Signalwegs wurde durch den quantitativen Nachweis der phosphorylierten, aktiven Form des CREB-Transkriptionsfaktors analysiert (Western Blot). Für die Bestimmung der Aktivität des Wnt-PCP-, des Wnt-Ca^{2+}- und des STAT-Signalweges wurden Transkriptionsfaktor Assays verwendet.

Im Einklang mit der Theorie, wonach eine monallelische β-catenin-Mutation im Codon 45 zu einer Akkumulierung von aktivem, dephosphoryliertem β-catenin führt, zeigt Aggr6 eine höhere basale Aktivität des kanonischen Wnt-Signaltransduktionsweges als die Zellkulturen normaler Fibroblasten. Während die geringe Menge des endogen produzierten WNT5A keinen Einfluss auf die Aktivität der kanonischen Wnt-Signalkaskade hat, führte die Zugabe von rekombinantem WNT5A zu einer deutlichen spezifischen Aktivitätssteigerung in der Tumorzellkultur Aggr6, nicht aber in Zellkulturen normaler Fibroblasten. Selektiv in Aggr6 führte die Stimulation mit rekombinantem WNT5A auch zu einer Aktivierung der nicht-kanonischen Wnt-Signaltransduktionswege Wnt-PCP und PKA. Durch den Einsatz entsprechender Inhibitoren und Aktivatoren für beide Signaltransduktionswege konnte gezeigt werden, dass beide Signalwege keinen Einfluss auf die Aktivität der kanonischen Wnt-Kaskade nehmen (kein „ Crosstalk“).

Ein Proliferationsassay ergab, dass rekombinantes WNT5A die Proliferationsrate in Aggr6 Tumorzellen steigert, auf Zellkulturen normaler Fibroblasten diesbezüglich aber keinen Einfluss ausübt. Eine Vorbehandlung der Zellen mit spezifischen Inhibitoren gegen den kanonischen Wnt- und die nicht-kanonischen Wnt-PCP- respektive PKA-Signalisationskaskaden mit anschliessender WNT5A Stimulation machte deutlich, dass der PKA-Signaltransduktionsweg sowohl für die WNT5A-induzierte Proliferationssteigerung, als auch für die basale Proliferationsrate der Aggr6-Tumorzellenellen von entscheidender Bedeutung ist.

Ein Typ I Kollagen-basierter Boyden-Chamber-Invasionsassay wurde für die Messung des invasiven Wachstumsverhaltens der einzelnen Zellkulturen herangezogen. Aggr6-Tumorzellen unterschieden sich in ihrer basalen Invasionsaktivität nicht von normalen Fibroblasten. Auch nach Zugabe von rekombinantem WNT5A zeigte sich keine Veränderung im Invasionsverhalten von Tumorzellen oder normalen Fibroblasten.

Das selektive Ansprechen der Aggr6-Tumorzellen auf eine Stimulation mit rekombinantem WNT5A konnte auch eindrücklich deutlich gemacht werden durch eine Analyse differentiell exprimierter Gene nach WNT5A-Stimulation mittels der Mikroarray-Technologie (Agilent). Die Anzahl der durch WNT5A in ihrer Expression

regulierter Gene überstieg in Aggr6 bei weitem diejenige in Kulturen normaler Fibroblasten.

Auf der Suche nach Gründen für die unterschiedliche Ansprechbarkeit von Aggr6-Tumorzellen und normalen Fibroblasten auf rekombinantes WNT5A wurde eine Analyse der Mikroarray-Expressions-Daten bezüglich der WNT5A-bindenden Frizzled-Rezeptoren vorgenommen. Es zeigte sich, dass Aggr6-Tumorzellen Fzd1, Fzd2 und Fz7 im Vergleich zu normalen Fibroblasten überexprimieren. Diese Rezeptoren sind in gleicher Weise auch im Tumorgewebe und in normalem Bindegewebe unterschiedlich exprimiert. Dies legte die Vermutung nahe, dass diese Rezeptoren für die Bindung von WNT5A an Tumorzellen verantwortlich sein könnten. Eine Inhibition der Expression dieser Frizzled-Rezeptoren mittels Transfektion der Aggr6-Zellen mit siRNA für alle drei Fzd-Rezeptoren führte aber nicht zu einer verminderten Ansprechbarkeit dieser Zellen gegenüber rekombinatem WNT5A.

Eine weitere Möglichkeit wäre, dass die in Aggr6-Tumorzellen vorhandene basale Aktivität der kanonischen Wnt-Signalisationskaskade die Expression von WNT5A-Rezeptoren induziert. Die Überexpression von an Codon 45 mutiertem β-catenin in normalen Fibroblasten führte zwar zu einer Aktivierung des kanonischen Wnt-Signaltransduktionswegs, ermöglichte aber keine weitere Aktivitätssteigerung durch anschliessende WNT5A-Stimulation. Der Grund für den unterschiedlichen Einfluss von WNT5A auf Tumorzellen aggressiver Fibromatosen gegenüber normalen Fibroblasten bleibt also unbekannt.

Zusammengefasst liefern die in vitro-Resultate die Grundlage zum Verständnis der Mechanismen, über welche WNT5A, das im Tumorgewebe aggressiver Fibromatosen konstant stark überexprimiert ist, seine proliferationsfördernde Wirkung ausübt.

.

The PhD-thesis is divided into three chapters dealing with different aspects of the experimental work:

Chapter I: Gene expression analysis of aggressive and superficial fibromatoses

Chapter II: Establishment of primary cell cultures derived from fibromatoses

Chapter III: The impact of WNT5A on cell signalling pathways and biological behaviour of aggressive fibromatosis tumor cells

Chapter I
Gene expression analysis of aggressive and superficial fibromatoses

1. General introduction

The discussions in subsequent sections of this chapter assume that basic signalling mechanisms and functions of the canonical Wnt-, the TGFβ- and the PI3K-AKT pathway are known, as well as basic principles of the process of wound healing. Therefore, those four themes should be introduced in this section.

1.1. Description of the canonical Wnt signalling pathway

1.1.1. Description (*Figure 1*)

The name "Wnt" is derived from the fusion of the names of two orthologous genes: the Drosophila gene Wingless (Wg) (Sharma and Chopra, 1976) and the mouse protooncogene Int-1 (Nusse and Varmus, 1982; Rijsewijk *et al.*, 1987). Wnt genes are coding for secreted glycoproteins that have been highly conserved during evolution, being expressed in species ranging from Drosophila to human (Giles *et al.*, 2003; Miller, 2002; van Amerongen *et al.*, 2008). Since their discovery more than 20 years ago, a plethora of studies has been performed in several organisms aiming to elucidate the signalling pathways elicited by the Wnts. The pathway that received by far the most attention represents the canonical Wnt pathway with its central component β-catenin. The description below is dedicated to this pathway. The non-canonical pathways will be introduced in C*hapter III, Section 1.1.*

In the absence of any Wnt-ligand binding to its receptor, newly synthesized β-catenin in the cytoplasm is bound by the two scaffold proteins axin (its binding affinity for β-catenin is increased after phosphorylation by the glycogene synthase kinase 3 β, GSK3β) and adenomatous polyposis coli (APC) (*Figure 1, (1)*), followed by the joining of the serine/threonine kinases GSK3β and casein kinase 1 α (CK1α). This multiprotein complex dedicated for the degradation of β-catenin in the cytoplasm is termed β-catenin degradation complex (Daugherty and Gottardi, 2007; Kimelman and Xu, 2006; Price, 2006; Willert and Jones, 2006).

Within this complex, β-catenin gets phosphorylated by CK1α at codon serine 45 (S45), followed by phosphorylations at the more N-terminally located residues threonine 41 (T41), serine 37 (S37) and serine 33 (S33) by GSK3β, whereby the first S45-phosphorylation represents an essential priming step for the subsequent phosphorylations at the other three residues (Amit *et al.*, 2002; Liu *et al.*, 2002; Yanagawa *et al.*, 2002). This process is accompanied by the simultaneous phosphorylations of APC by GSK3β and CK1α,δ,ε, leading to conformational changes within the degradation complex, resulting in an enhanced (APC) and reduced (axin) binding affinity for β-catenin, respectively (*Figure 1 (2)*). Consequently, N-terminally phosphorylated β-catenin is released from the degradation complex and APC acts to ensure its ubiquitination by the transducing repeat-containing protein β

(β-TrCP) ubiquitin ligase and its subsequent degradation by the proteasome (*Figure 1 (3)*).

In the absence of a functional APC protein, phosphorylated β-catenin is rapidly dephosphorylated by the serine/threonine protein phosphatase PP2A (Su *et al.*, 2008). APC is not essential for N-terminal phosphorylations of β-catenin by CK1α and GSK3β, since such phosphorylations can occur in APC mutant colon carcinoma cell lines (Yang *et al.*, 2006).

The canonical Wnt-signalling pathway gets activated by binding of a Wnt-ligand to its cognate receptor, a member of the Frizzled (Fzd) family of seven-span transmembrane receptors (Bhanot *et al.*, 1996; He *et al.*, 1997; Wang *et al.*, 1996), and co-receptor low-density lipoprotein receptor-related protein (LRP)-5 or -6 (Wehrli *et al.*, 2000). All three components – Wnt, Fzd and LRP – must be bound to each other in order to start the signalling pathway (Pinson *et al.*, 2000; Tamai *et al.*, 2000; Wehrli *et al.*, 2000) (*Figure 1 (4)*).

This leads (through a complex and as yet not fully understood mechanism (Daugherty and Gottardi, 2007; Kimelman and Xu, 2006; Price, 2006; Willert and Jones, 2006) involving the phosphorylations of LRP5/6 through GSK3β, CK1γ and CK1ε as well as of Dishevelled (Dsh) (Yanagawa *et al.*, 1995) through CK1ε to the sequestration of the β-catenin degradation complex components axin and GSK3β at the membrane. This results on one hand to the destabilization of the β-catenin degradation complex and a concomitant reduction of its ability to function, on the other hand to a direct inhibition and even degradation of components of the β-catenin degradation complex (Cselenyi *et al.*, 2008; Piao *et al.*, 2008; Wu *et al.*, 2009) (*Figure 1 (5)*). Whether by these mechanisms both CK1α mediated S45- and GSK3β dependent S33, S37, T41-phosphorylations are affected to a similar extent is controversially discussed in the literature (Amit *et al.*, 2002; Kimelman and Xu, 2006; Liu *et al.*, 2002).

However, as a net result of the activity of the canonical Wnt signalling pathway, N-terminally dephosphorylated β-catenin – at least at residues S33, S37 and T41 – accumulates in the cytoplasm (*Figure 1 (6)*), translocates into the nucleus and activates the transcription of T-cell transcription factor (TCF) / lymphoid enhancer-binding factor (LEF)-dependent target genes (*Figure 1 (7)*) (Molenaar *et al.*, 1996; Staal *et al.*, 2002; van de Wetering *et al.*, 1997; van Noort *et al.*, 2002). A comprehensive, updated overview of the canonical Wnt pathway target genes can be found on the Wnt homepage (*http:// www.stanford.edu /~rnusse /wntwindow. html*).

Whereas N-terminally phosphorylated β-catenin can be detected in the nucleus as well (Chung *et al.*, 2001; Clifford *et al.*, 2008; Nakopoulou *et al.*, 2006; Sadot *et al.*, 2002; Sokolova *et al.*, 2008), it does not contribute to the transactivation of TCF/LEF-dependent gene expression (Clifford *et al.*, 2008; Sadot *et al.*, 2002; Sokolova *et al.*, 2008; Staal *et al.*, 2002), since it is unable to form a ternary complex with target gene promoter DNA and TCF/LEF, although it is readily able to bind TCF/LEF (Clifford *et al.*, 2008; Sadot *et al.*, 2002). Therefore, the N-terminally dephosphorylated form of β-catenin is often called 'active'-β-catenin (van Noort *et al.*, 2002).

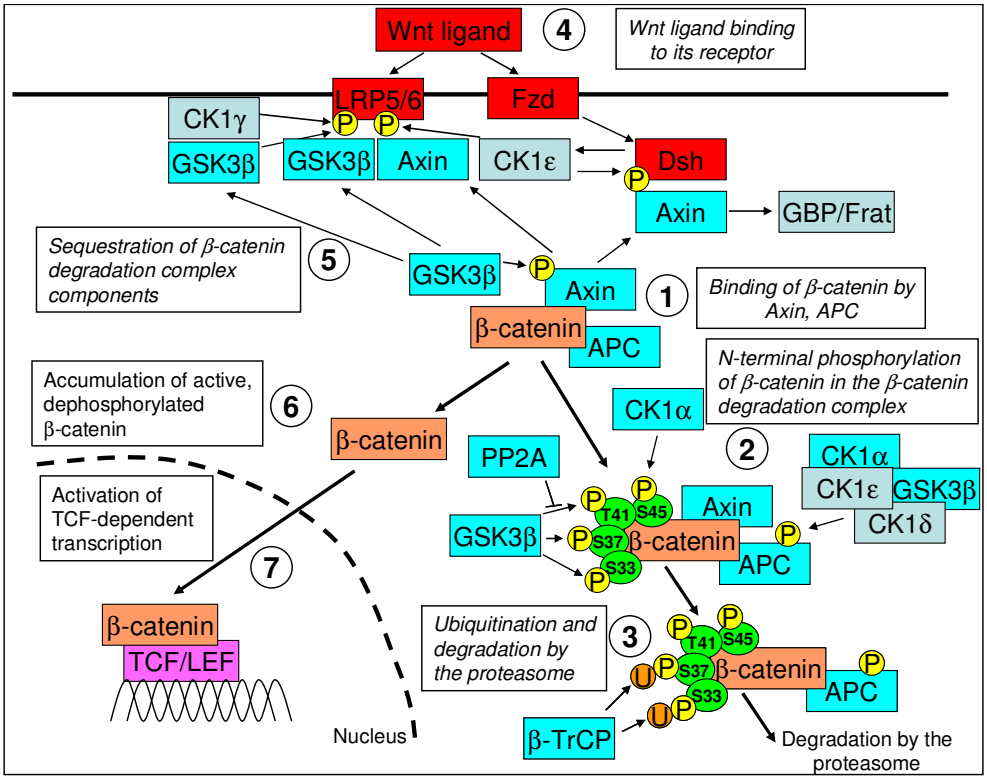

Figure 1: The impact of the canonical Wnt signalling pathway on the degradation of β-catenin. A detailed description is provided in the text.

β-catenin plays also a fundamental role in cell-cell adhesion, where it connects the transmembrane protein cadherin to the actin cytoskeleton (Daugherty and Gottardi, 2007). In this function, β-catenin is N-terminally dephosphorylated, since phosphorylations inhibit the interaction with its binding partners (Bustos *et al.*, 2006; Sadot *et al.*, 2002).

The above described mechanisms of phosphorylation and degradation of β-catenin form the basis of the 'classical model'. This view has been challenged by the observation that in genetically manipulated colon carcinoma HCT116 cells expressing only a S45-deleted β-catenin allele (-/Δ45-HCT116), phosphorylations at T41, S37 and S33 can readily occur (*Figure 2, red circle*), regardless of the missing phosphorylation at S45 (*Figure 2, blue circle*). This finding is clearly contradictory to the classical model, whereupon a S45 phosphorylation by CK1α represents an essential prerequisite for the subsequent phosphorylations at the other three residues by GSK3β (Wang *et al.*, 2003).

A recent paper demonstrated that the observed S33, S37, T41 phosphorylations in -/Δ45-HCT116 are not derived from GSK3β (Pai *et al.*, 2008). Nevertheless, phosphorylations at these residues are clearly reduced, since active, dephosphorylated β-catenin is present in higher amount, leading to an increased activity of the TCF/LEF-dependent transcription in the cell line -/Δ45-HCT116 (*Figure 3, red bars*) as compared to the cell line expressing only a wildtype β-catenin (*Figure 3, blue bars*) (Chan *et al.*, 2002). The discrepancy between the two models may relate to differences in regulation of β-catenin in different species or tissues or to different experimental approaches (e.g. exogenously introduced vs. endogenous β-catenin) (Wang *et al.*, 2003).

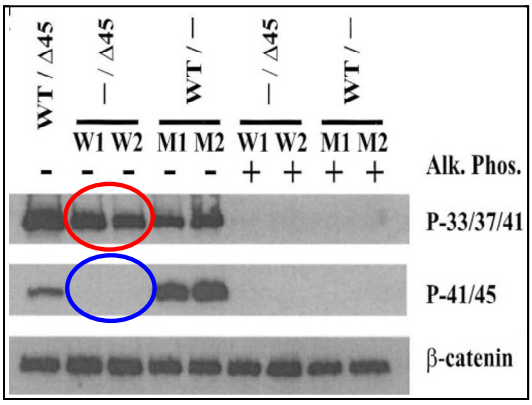

Figure 2: Observation challenging the classical model of phosphorylation and degradation of β-catenin: phosphorylations at T41, S37 and S33 can readily occur regardless of the missing phosphorylation at S45. W1,W2,M1,M2: different cell clones. Adapted from Wang *et al.*, 2003.

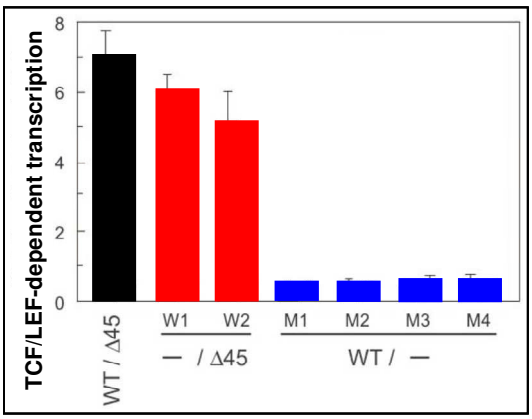

Figure 3: Regardless of the observed phosphorylations at S33, S37, T41, -/Δ45-HCT116 cells show an increased activity of TCF/LEF-dependent transcription as compared to the cell culture expressing a wild type β-catenin (WT/-). W1,W2,M1-M4: different cell clones. Adapted from Chan *et al.*, 2002.

1.1.2. The role of the canonical Wnt signalling pathway in embryogenesis and adult organisms

The canonical Wnt-pathway has been implicated in the regulation of virtually every aspect of embryonic development in all organisms analyzed, including axis specification, patterning, organogenesis, limb formation, adipogenesis and angiogenesis (Clevers, 2006; Moon *et al.*, 2002). In some self-renewing tissues in mammals, this pathway remains essential throughout life. For instance in the absorptive epithelium of the small intestine, in the hair follicle, the hematopoietic system or bone (Clevers, 2006).

1.1.3. The role of the canonical Wnt signalling pathway in tumorigenesis

The initial hint that the canonical Wnt signalling pathway leads to tumor formation when aberrantly activated came from studies in colorectal cancer cell lines.

The cell line SW480, carrying biallelic inactivating mutations in the tumor-suppressor gene APC, was characterized by a constitutively active TCF/LEF-dependent transcription that could be abrogated by an exogenous expression of APC (Morin *et*

al., 1997). This constitutive activation was shown to be dependent on the building of a β-catenin/TCF-4 complex, since exogenous expression of APC removed β-catenin from this complex and interfered with its transcriptional transactivation, indicating that inactivating APC-mutations inhibit the degradation process of β-catenin (Korinek *et al.*, 1997).

Sequencing of other colon carcinoma cell lines revealed that instead of biallelic mutations in APC (SW480), they carried monoallelic mutations in the N-terminal region of β-catenin at S45 (Δ45, HCT116) and S33 (S33Y, SW48), thus at those residues that are phosphorylated by CK1α and GSK3β in the multiprotein degradation complex. Transfection of human embryonic kidney cells (HEK293) with plasmids expressing β-catenin mutated at these residues revealed an enhanced activity of TCF/LEF-dependent transcription as compared to the activity in those cells transfected with a wildtype β-catenin expression plasmid (Morin *et al.*, 1997). Five years later, β-catenin mutations at those residues were shown to elicit an accumulation of β-catenin (Chan *et al.*, 2002) and active, dephosphorylated β-catenin (Staal *et al.*, 2002) in the nuclei of the cells analyzed, proving that mutations at these sites interfere with the phosphorylation events needed to target β-catenin for proteasomal degradation.

Since these initial findings, the summary of numerous reports analyzing the mutation status of APC and β-catenin in colorectal cancer led to the estimation that 90% of colorectal cancers are characterized by inactivating mutations in the tumor-suppressor gene APC (80%) or by activating mutations in the oncogene β-catenin (10%). β-catenin and/or APC mutations can also be found in tumors at other organs with variable frequency, such as hepatocellular carcinoma, gastric carcinoma or ovarian carcinoma (Giles *et al.*, 2003).

With axin, another component of the β-catenin degradation complex has been brough into context with the pathogenesis of cancer. Several studies reported mutations in this gene in ovarian carcinoma, hepatocellular carcinoma and medulloblastoma (Giles *et al.*, 2003).

Interestingly, there are only a few studies describing the involvement of Wnt ligands themselves in tumorigenesis. Ectopic expression of WNT1, WNT3A, WNT8 and WNT8B leads through accumulation of β-catenin to the transformation of mouse C57MG mammary epithelial cells, demonstrating their oncogenic potential in this cell culture (Wong *et al.*, 1994). Transgenic mice overexpressing WNT1 develop mammary adenocarcinoma (Tsukamoto *et al.*, 1988). In addition, the WNT2 gene was reported to be amplified in mammary tumors from mice (Nusse, 1992).

1.2. Description of the TGFβ pathway

1.2.1. Description (*Figure 4*)

The family of transforming growth factors (TGFβs) includes more than 30 factors that can be divided into two distinct groups (*Figure 4*). The first group compromises TGFβs themselves (TGFβ1, TGFβ2 and TGFβ3), activins, nodals, and myostatin, whereas the second is built up by bone morphogenic proteins (BMPs), anti-muellerian hormone (AMH) and various growth and differentiation factors (GDFs).

TGFβs are synthesized as a part of larger molecules, the pro-TGFβs, containing additionally the latency-associated proteins (LAP) which are cleaved from TGFβs in the Golgi apparatus but remain non-covalently bound to them. Extracellularly, latent TGFβ-binding proteins 1 and 2 (LTBP1/2) connect LAP-TGFβs to extracellular matrix (ECM) proteins. TGFβs can be released from this complex through the proteolytic activity of plasmin or matrix-metalloproteinases MMP2 and MMP9, or through integrins and thrombospondins (THBS). Two models are proposed how integrins contribute to the release of TGFβs: by simultaneously binding to both ECM-bound TGFβs and proteases, thereby improving the enzymatic cleavage, or by changing the conformation of ECM-bound TGFβ through the transmission of cell contraction forces. Thrombospondins associate with ECM-immobilized TGFβs and disrupt thereby the interaction between LAP and TGFβ.

Once released, TGFβs activate signalling by bringing together two pairs of receptor serine/threonine kinases known as type I and type II receptors. The type III receptor (also called betaglycan) is a membrane-anchored proteoglycan that supports the binding of TGFβ to the type II receptor. TGFβs themselves bind to TGFβ receptors, activins and myostatin to activin receptors, nodals to nodal receptors, whereas BMPs, GDFs and AMH bind to BMP receptors. Bound by a TGFβ, the two type II receptors phosphorylate and activate the two type I receptors that then propagate the signal by phosphorylating mothers against decapentaplegic homolog (Smad) transcription factors. Receptors of the first group of TGFβs (TGFβs, activins, myostatin, nodals) phosphorylate and activate Smad2 and Smad3, whereas those of the second group (receptors for BMPs, AMH, and GDFs) phosphorylate and activate Smad1, Smad5 and Smad8. These receptor-activated Smads are called receptor substrate Smads (RSmads).

After their activation, RSmads are characterized by a decreased affinity for the Smad-anchor for receptor activation (SARA), which in non-stimulated cells mediates the retention of the RSmads in the cytoplasm. Simultaneously, their affinity for Smad4 increases, leading to the building of a RSmads/Smad4 complex in the cytoplasm, its translocation into the nucleus and regulation of the transcription of various genes, including fibronectin (FN1), type I and III collagen, cyclin-dependent kinase inhibitors 1A (CDKN1A) or paired-like homeodomain transcription factor 2 (PITX2) (Massague, 2008; Pohlers *et al.*, 2009; Wipff and Hinz, 2008; Young and Murphy-Ullrich, 2004).

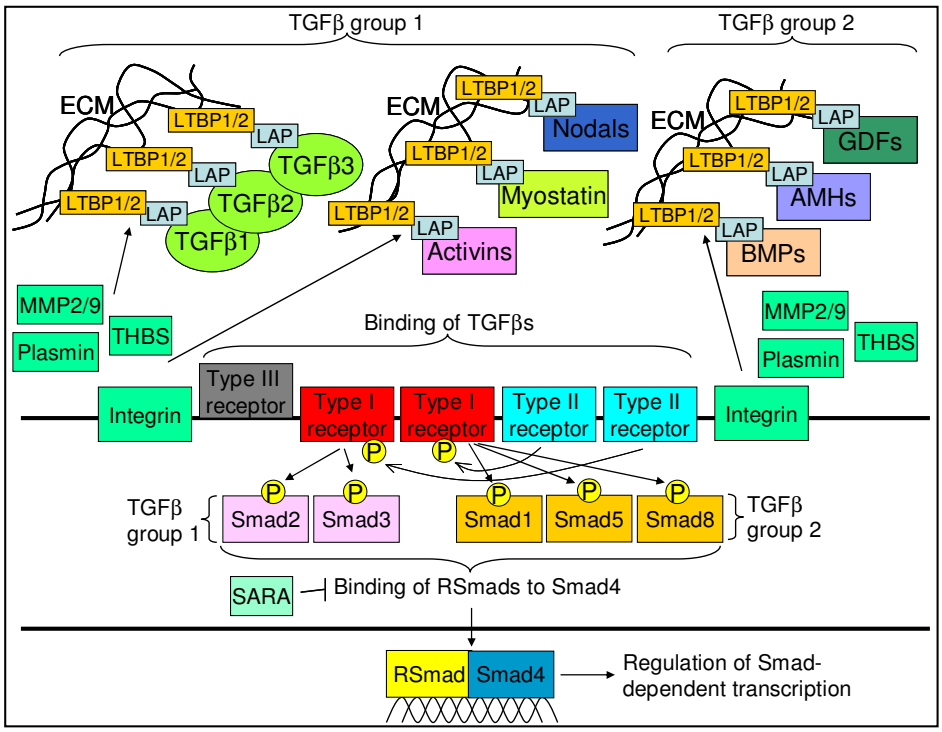

Figure 4: The TGFβ signalling pathway. A detailed description is provided in the text.

1.2.2. The role of the TGFβ signalling pathway in embryogenesis and adult organisms

TGFβ plays a crucial role in the following processes:

- Cell differentiation

TGFβ promotes the differentiation of mesenchymal precursors into fibroblasts and myofibroblasts at the expense of adipocytes and osteoblasts. On the other hand, BMPs promote the differentiation of mesenchymal precursors toward the osteoblast lineage (in bone growth and repair) and of neural precursors into astroglia. BMPs also stimulate the self-renewal of embryonic stem cells. Activins, nodals, BMPs, AMH and GDFs are important during embryogenesis for proper stem cell differentiation, body axis formation, left-right symmetry and organogenesis (Massague, 2008).

- Tissue homeostasis

TGFβ is involved in tissue homeostasis by controlling the secretion of growth factors and directly activating cytostatic mechanisms (Massague, 2008).

TGFβ was shown to constrain the secretion of growth factors such as hepatocyte growth factor (HGF) in stromal fibroblasts, thus reducing the paracrine stimulation of epithelial proliferation and inhibiting an epithelial hyperplasia in the prostate and forestomach (Bhowmick *et al.*, 2004).

It also directly exerts cytostatic effects on epithelial cells by inhibiting them to enter the synthesis (S)-phase of the cell cycle. TGFβ achieves that through transcriptional upregulation of the cyclin-dependent kinase inhibitors 1A (CDKN1A, p21; inhibits cyclin E/A – CDK2 complexes) and 2B (CDKN2B, p15; inhibits cyclin D - CDK4/6

complexes), the activation of CDKN1B (p27; inhibits inhibits cyclin E/A – CDK2 complexes), and the transcriptional repression of c-myc (MYC), a key transcriptional inducer of cell proliferation (Massague, 2008).

- Suppression of destructive immune and inflammatory reactions, promotion of immune tolerance

TGFβ is a key suppressor of destructive immune and inflammatory reactions, as proven by the lethal multifocal inflammatory disease arising in TGFβ1-deficient mice. As an immunosuppressive cytokine, TGFβ inhibits the development, proliferation and function of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, dendritic cells, natural killer cells and macrophages by the same cytostatic mechanism described for epithelial cells. In this way, TGFβ promotes immune tolerance that is especially important in the intestinal mucosa, where reactions to food antigens must be controlled. TGFβ1-deficient mice show chronic submucosal inflammatory reactions that frequently lead to colon carcinoma (Massague, 2008).

1.2.3. The role of the TGFβ signalling pathway in tumorigenesis

Under normal conditions, the antiproliferative effects of TGFβ described in the previous section counter the effects of local mitogenic stimulation to maintain tissue homeostasis. In tumors caused by oncogene expression, TGFβ induces proapototic mechanisms. These mechanisms include the increased expression of death-associated protein kinase 1 (DAPK1) and growth arrest and DNA damage inducible beta (GADD45B), which trigger apoptosis in a hepatoma cell line, and tumor necrosis factor receptor superfamily member 6 (TNFRSF6, FAS) as well as B-cell CLL/lymphoma 2 (BCL2)-like 11 (BCL2L11), which triggers apoptosis in gastric carcinoma cell lines (*Massagué 2008*).

Hence, cells must circumvent these tumor-suppressive (cytostatic and apoptotic) effects of TGFβ in order to be able to transform into cancer cells. Studies in different cancer types revealed that tumor cells can use two alternative ways to achieve that: 1) completely disrupt the TGFβ-signalling pathway by TGFβ receptor or Smad inactivating mutations or 2) selectively abrogate only the tumor-suppressive arm. By the latter mode, cancer cells may profit from the tumor promoting functions of TGFβ, e.g. its induction of production of mitogenic factors, its stimulating effect on epithelial-mesenchymal transition (EMT), or using it as a shield against anti-tumor immunity (Massague, 2008).

- Complete disruption of the pathway

Biallelic truncating or kinase domain inactivating mutations of the type II TGFβ receptor gene (TGFBR2) occur during tumorigenesis of colon, gastric, ovarian, esophageal and head and neck carcinomas. Such mutations are highly associated with microsatellite instability, pointing towards the occurrence of preceding mutations in mismatch repair genes during the progression of disease (Levy and Hill, 2006).

Whereas mutations in RSmads are infrequent in cancer, more than half of sporadic colorectal tumors and a high proportion of esophageal tumors carry mutations in Smad4 (Sjoblom *et al.*, 2006).

In mouse models, tissue specific inactivation of TGFBR2 or Smad4 alone is not sufficient to cause malignant epithelial cell transformation, but induces hyperplasia (Forrester *et al.*, 2005; Wang *et al.*, 2005). In contrast, mutations in these two genes strongly accelerate the malignant progression of tumors initiated by oncogenic stimuli. Inactivation of TGFBR2 induces the progression of adenomatous polyps initiated by biallelic APC mutations into carcinomas (Biswas *et al.*, 2004).

In addition to its direct growth inhibitory effect on epithelial cells, TGFβ can control their proliferation and tumor formation by repressing the production of paracrine growth factors in stromal fibroblasts and inflammatory cells. Mice with a deletion in the TGFBR2 gene in fibroblasts develop hyperplasia of the adjacent epithelia. This effect was accompanied by an increase in the expression of hepatocyte growth factor (HGF) in the mutation-carrying fibroblasts (Bhowmick *et al.*, 2004).

- Selective abrogation of the tumor-suppressive arm

Breast cancers, prostate cancers, gliomas, melanomas and hematopoietic neoplasias express normal TGFβ receptors and Smads, but are often characterized by a loss of function of the tumor-suppressive arm of the TGFβ signalling pathway (Massague, 2008).

In a study analyzing cultures of breast cancer cells from pleural fluids of patients with metastatic disease, the authors showed that the cells did not respond to the addition of TGFβ with an increased expression of CDKN2B and a reduction of the level of c-myc, despite retaining TGFβ induced growth factor expression. This was due to an overexpression of a dominant-negative form of a transcriptional co-activator / repressor of Smad that regulates the transcription of CDKN2B and c-myc (Gomis *et al.*, 2006).

Glioma cell cultures releaved from the tumor-suppressive effects of TGFβ increase their proliferation rate in response to TGFβ through the induction of platelet-derived growth factor B (PDGF-B) (Jennings and Pietenpol, 1998).

In another study, cultures of metastatic breast cancer cells did not respond as normally to TGFβ with a repressed expression of inhibitor of differentiation/DNA binding 1 (ID1), but instead showed an increased expression (Padua *et al.*, 2008). ID1 is known as being part of a lung metastasis gene expression signature and as being associated with relapse in estrogen receptor negative breast cancer cells (Minn *et al.*, 2005). A xenograft model in mice using human breast cancer cell lines showed that the expressions of ID1 and ID3 was essential for the successful formation of metastases after the cells entered the lung parenchyma (Gupta *et al.*, 2007).

In addition, cells having lost the tumor-suppressive arm of the TGFβ-pathway allow TGFβ to function as a potent inducer of the epithelial-mesenchymal transition (EMT), a well coordinated process during embryonic development and a pathological feature of tumors. Cells undergoing EMT lose the expression of E-cadherin and instead express mesechymal markers such as αSMA and vimentin and acquire motility and invasive capacity. TGFβ induces EMT by a combination of Smad-dependent transcriptional events and transcription-independent effects on cell-adhesion complexes (Massague, 2008).

1.3. Description of the PI3K-AKT signalling pathway

1.3.1. Description (*Figure 5*)

The phosphatidylinositol 3-kinase (PI3K) - v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway gets activated by binding of growth factors to their cognate receptor protein tyrosine kinases (RTKs) (*Figure 5*). RTKs include the receptors for the epidermal growth factor (EGFR and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, ERBB2), platelet-derived growth factor (PDGFR), fibroblast growth factor (FGFR), insulin-like growth factor 1 (IGF1R), vascular endothelial growth factor (VEGFR), hepatocyte growth factor (HGFR, MET) and interleukins (ILR).

Upon activation, RTKs interact and phosphorylate adapter proteins such as insulin receptor substrate (IRS) and growth factor receptor bound protein protein 2 (GRB2)-associated binding protein (GAB) that in turn activate PI3K. This kinase then phosphorylates membrane-bound phosphatidylinositol-3,4-diphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is an antagonist of PI3K that removes the phosphate group on PIP3, thus attenuating PI3K/AKT signalling activity. PIP3 binds to AKT and phosphoinositol-dependent kinase 1 (PDK1), whereupon PDK1 phosphorylates and activates AKT. An additional phosphorylation of AKT by PDK2 is required for its full activation.

Upon activation, AKT phosphorylates a number of downstream targets in the cytoplasm and the nucleus which regulate various cellular functions. It stimulates protein synthesis by phosphorylating the protein tuberin within the tuberous sclerosis complex 2 (TSC2) and by inhibiting the activity of the TSC1-TSC2 complex, leading to the accumulation and activation of the serine/threonine kinase mechanistic target of rapamycin (MTOR), which in turn phosphorylates target genes to enhance protein synthesis. It also stimulates glycogen synthesis by the phosphorylation and inactivation of GSK3β, known to phosphorylate and repress the activity of the glycogen synthase.

On the other hand, AKT stimulates cell proliferation via phosphorylation and inhibition of GSK3β and members of the forkhead box (FOXO) family of transcription factors. By inactivating GSK3β, AKT reduces GSK3β-mediated phosphorylation and inactivation of cyclin D1 (CCND1) and c-myc (MYC), two central components for cell cycle progression. In addition, inhibition of GSK3β leads to an accumulation of β-catenin and an activation of TCF-dependent transcription, whereupon the transcription of CCND1 and MYC is increased. Through the phosphorylation and inhibition of FOXO transcription factors, the PI3K-AKT pathway represses the expression of the cell cycle progression inhibitor cyclin-dependent kinase inhibitor CDKN1B (p27, inhibits cyclin E/A – CDK2 complexes), thus further accelerating proliferation.

Other target genes of the FOXO transcription factors repressed by the PI3K-AKT pathway represent the Fas ligand (FASLG), a member of the tumor necrosis factor super family (TNFSF6), and B-cell CLL/lymphoma 2 like 11 (BCL2L11), both known for their apoptosis-inducing capacity. Apoptosis gets further repressed by the AKT-dependent phosphorylation and activation of mouse double minute 2 homolog

(MDM2) that binds to and inhibits the key apoptosis-inducing transcription factor p53. Additionally, AKT phosphorylates and inhibits BCL2-associated agonist of cell death (BAD), an inducer of apoptosis through its inhibitory effect on the anti-apoptotic protein BCL2L1 (BCL-XL). Finally, AKT induces the activity of the anti-apoptotic signalling pathway nuclear factor of kappa light chain gene enhancer in B-cells (NFκB), by phosphorylating and activating the inhibitor of NFκB kinase (IKK) that in turn phosphorylates and inhibits the inhibitor of NFκB (NFKBI).

The PI3K-AKT pathway is also a potent inducer of angiogenesis. Hypoxia inducible factor 1 α (HIF1A) is the major regulator of vascular endothelial growth factor (VEGF) transcriptional activation. AKT induces the transcription of HIF1A via phosphorylation and activation of MDM2 and ribosomal protein S6 kinase polypeptide 1 (RPS6KB1) (Jiang and Liu, 2008).

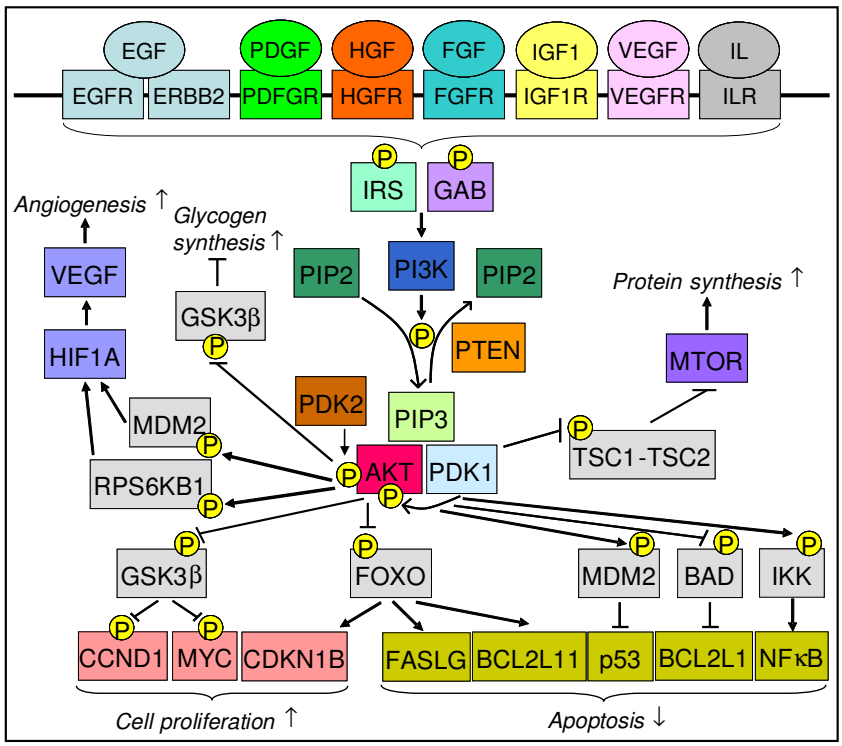

Figure 5: The PI3K-AKT signalling pathway. A detailed description is provided in the text.

1.3.2. The role of the PI3K-AKT pathway in embryogenesis and adult organisms

Due to its impact on central cellular processes such as proliferation, apoptosis, angiogenesis, protein synthesis and glycogen synthesis, the PI3K-AKT pathway plays a fundamental role in both embryogenesis and adult organisms (Suzuki *et al.*, 2008).

1.3.3. The role of the PI3K-AKT pathway in tumorigenesis

By positively influencing cell proliferation, angiogenesis and protein synthesis, but negatively regulating apoptosis, it is not surprising that a plethora of studies have associated a deregulated PI3K-AKT pathway activity with the pathogenesis of several cancers.
Most upstream of the pathway, gene alterations have been reported for EGFR, ERBB2 and IGF1R in glioblastoma, lung, ovarian, prostate, cervical endometrial, liver, esophageal, and breast cancers.

An amplification of the gene copy number of PI3KCA, the gene encoding the p110α catalytic subunit of PI3K, was observed with different frequencies in ovarian, cervical, gastric and breast cancers. In addition, somatic constitutively activating mutations of the PI3KCA gene were found in colorectal, gastric, breast and lung cancer, and in glioblastomas.

PI3K regulatory subunits dimerize with the catalytic subunits and inhibit PI3K activity. Deletions and somatic mutations of the gene PIK3R1 coding for the p85α regulatory subunit were found in glioblastoma, colon and ovarian cancers.

The antagonist of PI3K, PTEN, is frequently mutated or lost in glioblastomas, breast cancer, kidney and uterine endometrioid carcinomas, lung cancer and melanomas.

AKT gene amplification has been observed in a number of human cancers, including gastric carcinoma, glioblastoma, head and neck squamous cell carcinoma, pancreatic, ovarian, and breast cancers (reviewed by Jiang and Liu, *2008*).

1.4. The process of wound healing

Wound healing is a complex biological process that is dependent on interactions between a variety of cells, including fibroblasts, myofibroblasts, smooth muscle cells, endothelial cells, keratinocytes and immune cells. These interactions are orchestrated by numerous growth factors, including EGF, FGF, IGF, PDGF, TGF, VEGF and keratinocyte growth factor (KGF). TGFβ1 and platelet-derived growth factor (PDGF), released from platelets at the site of injury, provide the early signals for the activation and infiltration of macrophages and neutrophils. These cell types in turn secrete the next wave of growth factors essential for the proliferative phase of wound healing. This phase is characterized by an increased proliferation of fibroblasts, their transformation into myofibroblasts and invasion of the wounded tissue. There, they deposit an abundant collagen-rich matrix needed to support further cell ingrowth and to stabilize and contract the wound (granulation tissue).

TCF-dependent transcription plays a crucial role in the proliferative phase of wound healing. Wounding of a transgenic TCF-reporter mouse (TCF-reporter construct containing the lacZ gene downstream of a c-fos minimal promoter and three consensus TCF-binding motifs) revealed an increased expression of β-catenin (Western blot) and activity of TCF-dependent transcription in the proliferating fibroblasts during the proliferative phase of wound healing as compared to fibroblasts in unwounded tissues. Wounding of a transgenic mouse expressing a tetracyclin-inducible N-terminal mutated β-catenin (S33, S37, T41, S45 to A, thus resistant to CK1α and GSK3β-mediated phosphorylation and degradation by the proteasome) leads to the formation of a larger, hypercellular wound characterized by an excessive collagen deposition and a higher fibroblast proliferation rate, as compared to the wound in a wildtype-β-catenin animal (Cheon *et al.*, 2002). Analyses of human re-excision scar tissue after a previous biopsy and samples from hyperplastic cutaneous wounds revealed increased levels of α-SMA, type III collagen (RT-PCR) and β-catenin (Western blot) as compared to normal surrounding tissues. β-catenin was shown to be located in the cytoplasm and nucleus (IHC) and correlated with the level of S9-phospho-GSK3β, the functionally inhibited form of GSK3β (Western blot). The expression of fibronectin and matrix-metalloproteinase 7 (MMP7), two known target genes of the canonical Wnt signalling pathway, were elevated as compared to the expression in the surrounding normal tissues (RT-PCR) (Cheon *et al.*, 2005).

Dermal fibroblast cell cultures of the TCF-reporter mouse revealed an increased expression of β-catenin and S9-phospho-GSK3β (Western blot) and an enhanced activity of TCF-dependent transcription after treatment with TGFβ1 or EGF. Subcutaneous injection of TGFβ1 or EGF before wounding of the TCF-reporter mouse led to an increased activity of TCF-dependent transcription in the proliferating fibroblasts during the proliferative phase of wound healing resulting in a larger wound as compared to the situation after wounding of an untreated TCF-reporter mouse (Cheon *et al.*, 2004). Those data suggest that TGFβ1 and EGF induce the proliferative phase of wound healing by transactivating the canonical Wnt signalling pathway.

2. Introduction

In the following section, a general clinical, histological and phenotypical description of fibromatoses will be provided, in addition to an introduction into the signalling pathways known to be involved in the pathogenesis of these diseases. Finally, some results of published gene expression analyses aimed to compare fibromatoses with normal fibrous tissues will be presented.

2.1. General description

Fibromatoses are invasively growing fibrous lesions lacking the capacity to metastasize. Depending on the sites of involvement, they are categorized as aggressive and superficial fibromatoses. Aggressive (synonyms: desmoid-type or deep) fibromatoses occur extra- and intra-abdominally in the connective tissues surrounding muscles, whereas the predilection sites for superficial fibromatoses are the palmar and plantar aponeuroses.

The pathogenesis of aggressive fibromatosis is a proven neoplastic process derived from a single cell that acquired a growth advantage (Alman *et al.*, 1997b; Li *et al.*, 1996). In contrast, whether superficial fibromatosis represents a clonal neoplastic process or rather a polyclonal reactive process is controversially discussed. Two studies report a polyclonal pattern of X chromosome inactivation of the androgen receptor gene in tissues from two respectively eight women with superficial fibromatosis, thus favoring a polyclonal reactive process (Chansky *et al.*, 1999; Wang and Zhu, 2006). Other papers describe clonal chromosomal aberrations at variable frequencies in patients suffering from this disease, supporting the notion of a neoplastic process (Dal Cin *et al.*, 1999; De Wever *et al.*, 2000).

Histologically, the tumors are characterized by spindle-shaped cells embedded in an abundant collagen-rich extracellular matrix (*Figure 6*). The tumor cells exhibit features of myofibroblasts as indicated by the expression of bundles of smooth muscle actin (α-SMA) microfilaments similar to those found in smooth muscle cells. For the superficial fibromatoses, three distinct histological phases can be distinguished. In the proliferative phase, a nodular lesion develops through the proliferation of myofibroblasts that express α-SMA. In the involutional phase, the cells within the nodule realign themselves with the lines of stress in the tissue, therefore building cords. The residual phase corresponds to the complete disappearance of the nodules, leaving behind cords of relatively acellular, scar-like tissue (*3*).

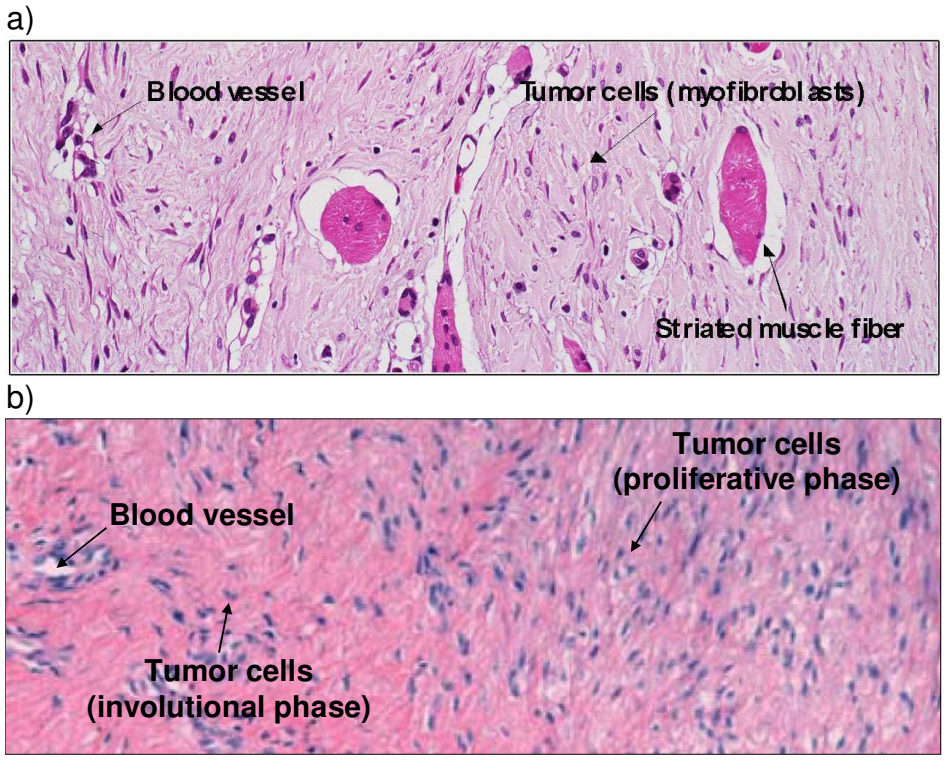

Figure 6: Histology of an aggressive (a) and superficial (b) fibromatosis. Hematoxylin-Eosin (HE)-staining.

Numerous reports in the literature using different experimental techniques such as gene expression array, immunohistochemistry, in situ hybridization or Western blot describe an overexpression of a vaste array of different components of the extracellular matrix (ECM) in fibromatoses.

There are only a few etiologic factors contributing to the pathogenesis of aggressive fibromatoses, namely surgical or physiological trauma and radiation. For superficial fibromatoses, an association with trauma, alcohol, smoking, manual work, diabetes mellitus, epilepsy and rheumatoid disease has been described.

2.2. Signalling pathways known to be involved in the pathogenesis of fibromatoses

2.2.1. The canonical Wnt signalling pathway

- Data derived from primary tumors

Aggressive fibromatoses occur as sporadic lesions (80%) or as part of the disease familial adenomatous polyposis (FAP, 20%), an inherited disorder characterized by the development of adenomatous polyps of the colon (Tejpar *et al.*, 2005). Extracolonic manifestations of FAP include, among others, aggressive fibromatosis. FAP is caused by a germ-line mutation in the tumor suppressor gene APC (Kinzler *et al.*, 1991). The role of APC in the canonical Wnt pathway has been described in *Section 1.1.1.* Polyps and aggressive fibromatosis in FAP patients show a biallelic inactivation of the APC gene, by additionally acquiring a somatic mutation on the second allele. In sporadic aggressive fibromatoses, biallelic inactivation of the APC

gene by somatic mutations was found in ~20% of cases (Tejpar *et al.*, 1999). In addition, ~52% to ~85% of the reported cases of sporadic aggressive fibromatoses demonstrate monoallelic mutations in the oncogene β-catenin (Lazar *et al.*, 2008; Tejpar *et al.*, 1999), whereas ~26% of cases remain as yet unexplained (Tejpar *et al.*, 1999).

Immunohistochemical analysis of β-catenin expression however, revealed increased cytoplasmic expression and nuclear accumulation in most if not all aggressive fibromatoses analyzed, regardless of their mutation status (Alman *et al.*, 1997a; Carlson and Fletcher, 2007; Ferenc *et al.*, 2009; Lazar *et al.*, 2008; Montgomery *et al.*, 2001; Saito *et al.*, 2001; Tejpar *et al.*, 1999). Thus the cause for this phenomenon in the remaining ~26% of sporadic tumors remains to be elucidated (*Figure 7*).

Like aggressive fibromatoses, also superficial fibromatoses show increased cytoplasmic and nuclear accumulation of β-catenin (Carlson and Fletcher, 2007; Montgomery *et al.*, 2001; Varallo *et al.*, 2003), but neither β-catenin (Montgomery *et al.*, 2001; Varallo *et al.*, 2003) nor APC (Montgomery *et al.*, 2001) is mutated in these tumors. The etiology of this tumor is unknown (*Figure 7*).

	APC mutation	β-catenin mutation	No mutation	β-catenin↑ nucleus
Aggressive fibromatosis FAP-assoc. (20%)	100% biallelic	-	-	√
Aggressive fibromatosis sporadic (80%)	~20% biallelic	~52-85% monoall.	~26%	√
Superficial fibromatosis	-	-	100%	√

Figure 7: APC and β-catenin mutation status of fibromatoses

The intensity of nuclear β-catenin expression in sporadic aggressive fibromatoses was shown to inversely correlate with the incidence of recurrence after surgical resection (Lazar *et al.*, 2008). In sporadic aggressive fibromatoses caused by β-catenin mutations, ~59% occur at codon 41 (T41A) and ~41% at codon 45 (33%: S45F; 8%: S45P), both known target sites for CK1α (S45) and GSK3β (T41) mediated phosphorylation. Interestingly, the five-year recurrence-free survival was significantly poorer in 45F-mutated desmoids (23%) versus either 41A (57%) or non-mutated tumors (65%) (Lazar *et al.*, 2008).

- Data derived from cell cultures (aggressive fibromatosis)

That aberrant canonical Wnt signalling pathway activity contributes to the formation of aggressive fibromatosis was underlined by experiments performed in primary cell cultures derived from this tumor. In the following studies, the authors did not prove the tumoral origin of their cell cultures used by mutation analysis.

In a first report, the experiments were based on cell cultures derived from aggressive fibromatoses carrying somatic APC mutations. Transient transfection of these cells

with an APC expression plasmid led to a decrease of the β-catenin protein level (Western blot) and a diminished proliferation rate. A concomitant expression of a constitutive active form of β-catenin (ΔN89-β-catenin, missing the N-terminal phosphorylation sites) abrogated the negative effect of APC on the cellular proliferation, indicating that APC controls the proliferation of these cells through its contribution to the degradation process of β-catenin (Li *et al.*, 1998). In a subsequent study, 6 primary cell cultures of aggressive fibromatoses (tumor tissues: 3x β-catenin mutation, 1x biallelic APC-mutation, 2x no APC/β-catenin mutation) were reported to be characterized by a pronounced nuclear accumulation of β-catenin in comparison to normal control fibroblasts (Tejpar *et al.*, 1999). In a further study, the authors claim that primary cell cultures from aggressive fibromatoses carrying APC- and β-catenin-T41A-mutations, respectively, are characterized by an increased TCF-dependent transcriptional activity as compared to cell cultures of normal fibroblasts. In addition, they could show that in aggressive fibromatoses, β-catenin transactivates TCF-dependent transcription by binding to TCF3, not TCF4, thus contrasts to the situation in colon carcinoma (Tejpar *et al.*, 2001).

- Data derived from animal models (aggressive fibromatosis)

Two mouse models further prove the context of a deregulated canonical Wnt-signalling pathway and the formation of aggressive fibromatoses. In the first, heterozygous APC+/APC1638N animals develop aggressive fibromatoses in addition to attenuated polyposis (Smits *et al.*, 1998). In the other model, transgenic mice expressing a tetracyclin-inducible mutated β-catenin (S33, S37, T41, S45 to A) develop in 75% of the cases aggressive fibromatoses after 3 month of transgene induction, in addition to gastrointestinal polyps in all mice. Primary fibroblast cultures of transgenic mice revealed an increased proliferation, motility and invasiveness as compared to normal fibroblasts and are able to form tumors in nude mice (Cheon *et al.*, 2002).

2.2.2. The TGFβ signalling pathway (superficial fibromatosis)

In contrast to aggressive fibromatosis a vast literature exists describing the involvement of the TGFβ signalling pathway in the pathogenesis of superficial fibromatosis.

- Data derived from primary tumors

Immunohistochemical analyses revealed an overexpression of TGFβ1 and TGFβ2 in tissues of superficial fibromatoses as compared to normal palmar fasica (Badalamente *et al.*, 1996; Berndt *et al.*, 1995), a finding that was confirmed for TGFβ2 using microarray gene expression analyses (Lee *et al.*, 2006; Zhang *et al.*, 2008).

An attempt to correlate single nucleotide polymorphisms (SNPs) in the genes coding for the three TGFβ receptors with the risk of superficial fibromatosis formation provided no conclusive evidence for their contribution to the pathogenesis of superficial fibromatoses (Bayat *et al.*, 2003a). In a subsequent study, the same author found a significant correlation between a single SNP (a guanine instead of an adenine in the 3' untranslated region) in the gene coding for the transcription factor

kruppel-like factor 6 (KLF6) and an increased risk of developing superficial fibromatosis. Since KLF6 is known for its positive impact on the expression of TGFβ1, the observed SNP may contribute to a deregulated TGFβ signalling pathway activity leading to the formation of superficial fibromatosis (Bayat *et al.*, 2003b).

- Data derived from cell cultures

In primary cell cultures derived from superficial fibromatoses, the expression of TGFβ2 was shown to be upregulated as compared to normal control fibroblasts (ELISA) (Kuhn *et al.*, 2002).

A positive effect of TGFβ1 and TGFβ2 on the proliferation of superficial fibromatosis cell cultures was also reported, whereas cultures of normal fibroblasts did not react (Badalamente *et al.*, 1996). This result was indirectly proven by an experiment showing that TGFβ2 increases the DNA and protein synthesis in cultures of superficial fibromatoses (Kuhn *et al.*, 2001).

TGFβ1 enhanced the proportion of αSMA-positive myofibroblasts in cultures of superficial fibromatoses, whereas normal fibroblast did not respond (IHC) (Bisson *et al.*, 2003). In another cell culture experiment, TGFβ1 was shown to induce the expression of αSMA, type I collagen and plasminogen activator inhibitor type I (PAI-1) (Western blot) (Kopp *et al.*, 2006).

Primary tumor cells of superficial fibromatoses contracted fibroblast-populated collagen lattices (FPCL) significantly more than control fibroblasts (Kuhn *et al.*, 2002). The addition of TGFβ1 (Bisson *et al.*, 2009) and TGFβ2 (Tse *et al.*, 2004) increased the generated force in both control and diseased cells.

The above listed results demonstrate that TGFβ is able to induce in normal fibroblasts all features that characterize superficial fibromatoses: enhanced proliferation, differentiation into myofibroblasts, increased production of extracellular matrix proteins and increased contractile force, pointing towards a decisive role of the TGFβ signalling pathway in the pathogenesis of superficial fibromatosis.

In all reports describing experiments with primary cell cultures from superficial fibromatoses, a formal proof for the tumoral origin of the cell cultures used was not provided.

- Data derived from animal models

The TGFβ-dependent induction of extracellular matrix proteins was also analyzed in nude rats implanted with human superficial fibromatosis tissue pieces. Explanted tissues perfused with TGFβ2 showed an increased synthesis of type-I and type-III collagen (IHC) as compared to tissues perfused with a vehicle control (Kuhn *et al.*, 2001).

2.3. Microarray-based gene expression analyses

Some microarray-based gene expression studies have been performed to compare the gene expression of aggressive and superficial fibromatoses with the one in reference fibrous tissues. An overview of numerously represented biological functions of the differentially expressed genes should be provided for each study in this section. A more detailed description of these reports will be supplied in *Section 5.2.2.*

2.3.1. Aggressive fibromatosis

In one study, the gene expression of four primary cell cultures of aggressive fibromatoses was compared with the one in cultures of normal fascia using Affymetrix microarrays. They report the differential expression of components of biological processes known to be involved in the pathogenesis of cancer, such as the overexpression of genes regulating proliferation and proteolysis of extracellular matrix (ECM) components (Denys *et al.*, 2004b).

In a second study, 12 tissue samples of aggressive fibromatoses were compared with 16 non-neoplastic tissues, including normal adipose, cervix, colon, kidney, liver lung, using Affymetrix-based gene expression analysis. Among the upregulated genes several ECM components and growth factors could be found (Skubitz and Skubitz, 2004).

2.3.2. Superficial fibromatosis

On Atlas microarrays, the gene expression of 6 tissues of palmar superficial fibromatoses was compared with the one in 2 unaffected palmar fascia. Some of the differentially expressed genes are involved in the ECM and in cell-cell adhesion (Pan *et al.*, 2003) (Pan *et al.,* 2003).

Using the same Atlas microarrays, the authors of another study compared tissues of 9 palmar nodular superficial fibromatoses with adjacent normal tendon. ECM components and genes involved in collagen degradation could be found among the upregulated genes (Qian *et al.*, 2004).

On cDNA arrays, tissues derived from 4 palmar cord superficial fibromatoses, four adjacent control fascia and three control palmar fascia were compared with each other. Among the upregulated genes in superficial fibromatoses, some ECM components and genes regulating proliferation could be detected (Lee *et al.*, 2006).

The gene expression in 6 primary cell cultures derived from palmar superficial fibromatoses was compared with the one in 6 normal palmar fascia fibroblast cell cultures using 2 different microarray platforms (GE Code LinkTM and IlluminaTM). Some ECM components were downregulated in diseased cultures on both platforms (Satish *et al.*, 2008).

Another cDNA microarray analysis compared the gene expression of palmar cord superficial fibromatosis tissues with the one in adjacent control fascia. Differentially expressed genes represent ECM components and regulators of proliferation (Zhang *et al.*, 2008).

2.4. Aim of the study

The aim of the experiments described in this chapter was to study the differential gene expression between primary tissues of aggressive and superficial fibromatoses and reference fibrous tissues using microarray analyses. Whereas microarray-based gene expression studies between fibromatoses and reference tissues and between corresponding cell cultures have already been published in the literature (*Section 2.3.*), a direct comparison between those two phenotypically and histologically similar tumors has not been performed, yet. Therefore, this study, in addition to reveal new deregulated genes in aggressive and superficial fibromatosis, should contribute to the understanding of common and distinctive mechanisms being responsible for the pathogenesis of aggressive and superficial fibromatoses.

3. Materials and methods

3.1. Collection and characterization of fibromatoses and reference fibrous tissues

3.1.1. Tissue asservation

Tissue samples used in this study were obtained immediately after surgery or at autopsy and flash frozen in liquid nitrogen. They were stored in Hank's balanced salt solution at -80°C. Two types of dense connective tissues were used as samples for the reference fibrous tissue pool: fascia lata (1 sample) and patellar ligament (4 samples). They were derived from patients without fibromatoses. For the preparation of paraffin blocks, pieces of tissues were fixed in 4% buffered formalin (pH 7.0) for at least 24 hours. Subsequently, they were dehydrated and embedded in paraffin using the TBS88 Paraffin Embedding System TBS88 (Medite, Nunningen, Switzerland).

3.1.2. Histology

For histological examination, paraffin sections were cut and stained with hematoxylin-eosin (H&E) in an automated staining system (Tissue stainer COT20, Medite, Nunningen, Switzerland). Histological diagnosis was performed by two independent pathologists. Additionally, all cases were reviewed before their use in this study. All tumors exhibited the characteristic histological picture of fibromatoses (*Section 2.1.*, *Figure 6*).

3.2. Agilent 60mer-oligo microarrays

3.2.1. Total RNA extraction

Total RNA was extracted from cryopreserved tissues using the TriReagent (Molecular Research Center, Inc., Cincinnati, OH) protocol. Frozen tissue specimens were cut into 100 µm sections on a cryostat and maintained on dry ice. A total of ~ 25 sections were homogenized in 4 ml of TriReagent solution using a Rotor Stator homogenizer. The first, the middle and the last section were stained with H&E to monitor the composition of the samples (exclusion of those with exuberant vascularisation or fat tissue). Resulting total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Agilent Technologies, Santa Clara, CA).

3.2.2. RNA quality control

The integrity and purity of the extracted total RNA was monitored using the RNA 6000 Nano Kit on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Only samples characterized by a 28S:18S rRNA ratio of greater than 1.0 and a flat baseline on the electropherogram above and below the 18S and 28S rRNA peaks were further used.

3.2.3. Experimental setup

Equal amounts of total RNA derived from 10 individual aggressive fibromatoses (*Figure 8*, framed red circles) and 15 individual superficial fibromatoses (framed blue circles) were pooled to generate an aggressive fibromatosis RNA pool and a superficial fibromatosis RNA pool. As a reference, RNA samples from five fibrous tissues were pooled (framed black circles). In addition, RNA samples derived from 5 single aggressive fibromatoses and 1 single superficial fibromatosis were hybridized on individual arrays (additional red and blue circles).

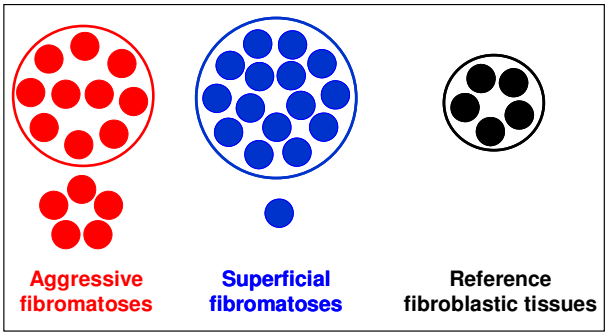

Figure 8: Experimental setup for the Agilent microarray gene expression analyses

3.2.4. Labelling, hybridization and data processing

44K Agilent 60mer-oligo micorarrays (Agilent Technologies, Santa Clara, CA) were used in this study. Instead of annealing the oligos fully synthesized onto the microarray surface, they are synthesized base-by-base in microdroplets using phosphoramidite chemistry. In phosphoramidite synthesis reactions, the reactive sites on the nucleotides are blocked with chemical groups that can be removed selectively. This allows to add one base at a time to the oligo chain in a single droplet in a very controlled manner. After the first base is printed, the chemical group that protects the 5' hydroxyl group on the nucleotide is removed and oxidized to activate it, enabling it to react with the 3' group on the next nucleotide. In between each step, the excess reagents are washed away. This process of 'printing' a nucleotide followed by washing, the remove of the protecting chemical group and the addition of the new nucleotide is repeated 60 times to synthesize the 60mer-oligos present on these microarrays. ~41'000 60mer-oligos are synthesized on each array, representing over 33'000 known and unknown human genes.

The first round of experiments involving the pooled RNA samples were performed using the dual color labelling and competitive hybridization method. This approach reduced experimental variability by allowing two biological samples to be directly compared with each other on the same microarray.

The second round of experiments aimed to analyze the six individual tumor samples was performed after the one color labelling and hybridization technology was improved, newly including the RNA Spike-In feature for monitoring the quality of the workflow from sample amplification and labelling to the final data processing. For each array, a quality control report could be generated based on the Spike-In informations. This allows to obtain data that are even more reliable and comparable then the ones generated with the dual color method. To be able to compare data

generated by the two different methods, the pooled RNA samples were analyzed by the one color approach as well.

- Dual color labelling, competitive hybridization and data processing

The necessary experimental steps for the dual color labelling reaction were performed according to the 'Agilent Low RNA Input Fluorescent Linear Amplification Kit, Version 2.0 (August 2003)' protocol, whereas for the hybridization procedure, the instructions were obtained from the 'Agilent 60-mer oligo microarray processing protocol, Version 2.1 (April 2004)' (Agilent Technologies, Santa Clara, CA). *Figure 9* depicts the relevant experimental steps. Briefly, after the reverse transcription of the mRNA (0.75 µg total RNA) into cDNA and the production of the second cDNA strand, probes were labelled by an in vitro transcription step using different fluorescent dye-labeled CTPs for the two samples to be compared, namely Cy3-CTPs and Cy5-CTPs. 1 µg of each labelled cRNA was simultaneously hybridized on an Agilent 44K 60mer oligo microarray. A spot with a light green signal indicates exclusive binding of Cy3-labeled cRNA, a light red Cy5-labeled cRNA binding, and a light yellow signal arises from an equivalent mixture of the two. Microarrays were scanned using the DNA Microarray scanner (Agilent Technologies, Santa Clara, CA). The Feature Extraction Software v8.1 (Agilent Technologies, Santa Clara, CA) was used to calculate the ratio of the signals Cy5/Cy3 for each spot to detect differentially expressed genes between the two samples. Additionally, this software delivers for each spot a specific p-value, indicating the trustability of the calculated differential gene expression. This p-value is based on the ratio Cy5/Cy3 and on a platform-specific error model that simulates all the errors that can occur from the initial reverse transcription to the final calculations. Further data processing, including selection of differentially expressed genes to be further analyzed and hierarchical clustering, was performed using the Rosetta Resolver software (Rosetta Inpharmatics LLC, Seattle, WA). For functional annotation of selected genes, the software Database for Annotation, Visualization and Integrated Discovery (DAVID; *http:// david.abcc.ncifcrf.gov*) was used.

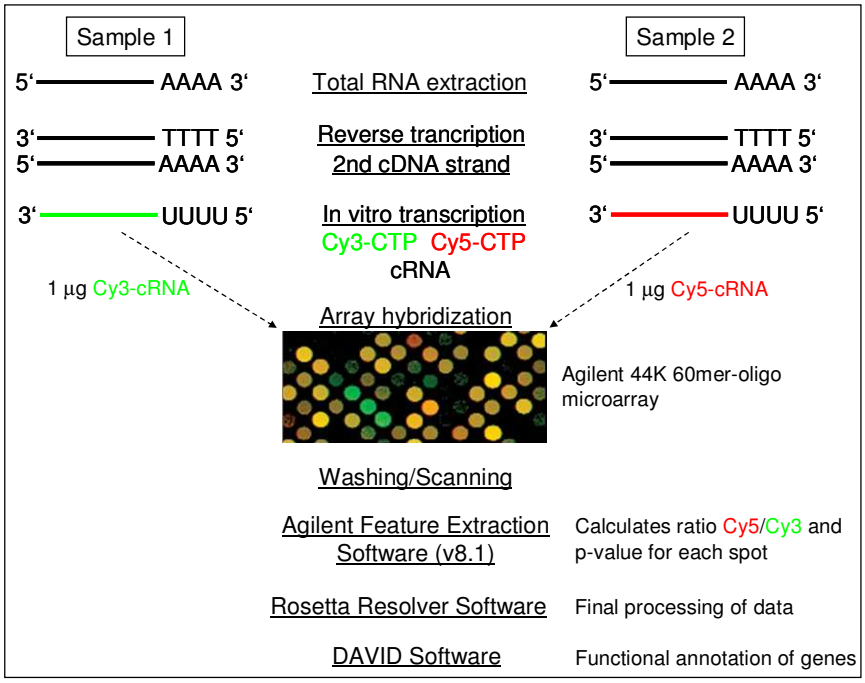

Figure 9: Work flow for dual color labelling, competitive hybridization, and data processing of Agilent 44K 60mer-oligo microarrays

- One color labelling, hybridization and data processing

The basic principles for this approach are the same as outlined for the dual color method, with the difference, that one color (Cy3)-labelled cRNA of just one single sample was hybridized per array. In comparison to arrays used for competitive hybridization, these slides contained four single microarrays, therefore their name '4x44K 60mer-oligo microarray' (Agilent Technologies, Santa Clara, CA). The experimental procedure followed the 'One color microarray-based gene expression analysis, Version 1.0.1 (February 2006)' protocol obtained from the company. Instead of the Agilent Feature Extraction Software version 8.1, the new version 8.5 (Agilent Technologies, Santa Clara, CA) was used, that allows to generate the quality control report. Again, the final processing of data was performed on the Rosetta Resolver software and the functional annotation of selected genes using the software DAVID.

3.3. Real-time reverse transcription PCR (RT-PCR)

Total RNA was isolated from cryopreserved tissues as mentioned in *Section 3.2.1.* and its quality determined according to the description in *Section 3.2*.2. Equal amounts (5 µg) were reversely transcribed into cDNA with the oligo$(dT)_{18}$ primer and Transcriptor Reverse Transcriptase using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The Roche LightCycler 480 system with 384 well plates was used (Roche, Basel, Switzerland). Amplification of a selected gene was based on the use of the Human Universal Probe Library (Roche, Basel, Switzerland) that is build up by 90 different fluorescein-labeled probes. Together with two gene specific primers (Mycrosynth GmbH, Balgach, Switzerland) those 90 probes cover 99% of the human transcriptome. For each gene analyzed, a standard-curve was calculated first, using 50, 10, 2, 0.4, 0.08 and 0.016 ng input cDNA. Based on these standard-curves, their relative expressions to the house-keeping gene GAPDH were determined in each sample using 10 ng input cDNA. A reaction (20 µl) included: 5 µl (2 ng/µl) cDNA, 0.1 µl forward primer (100 µM $\rightarrow$ 0.5 µM final concentration), 0.1 µl reverse primer (100 µM $\rightarrow$ 0.5 µM final concentration), 0.2 µl probe (10 µM $\rightarrow$ 0.1 µM final concentration), 10 µl LightCycler 480 Probes Master (Roche, Basel, Switzerland) and 4.6 µl H_2O. The probe number and corresponding primer sequences for each gene analyzed were derived from the free-access, web-based ProbeFinder Software (www.universalprobelibrary.com). *Table 1* lists up the Universal Probe Library number and corresponding forward and reverse primer sequences for all genes analyzed by real-time RT-PCR.

Gene	Probe #	Forward primer	Reverse primer
ANGPTL7	44	TGTATAAGCTTCCTCCTGATGACTT	CGCCTGAAGTCTCCATGTC
APCDD1	90	CACCGAGTTCGTGTTCAAAG	TCCCGTTGAAGACGTTGAG
ASPN	72	CATGGTGGATAACTTCTACTTTTAGGA	GAAGGGTTTGGCAGAGCAC
CDC73	33	CAAATGCAGCACCTGTGG	CCTTTGAATCTTTCCTGATCGT
CTHRC1	57	GGGAGGTGGTGGACCTGTA	CAGGTGTACCCGGAATGC
DCD	43	AGCCCTGGTCTGTGCCTAT	GCTTCATGGCAAGGGTTC
DKK3	29	TATCACATCTGTGGGAGACGAA	TCCTCGTCGATGATGCACT
FGF18	60	ATGAACCGCAAAGGCAAG	GAACACACACTCCTTGCTGGT
FRZB	31	TCATGGGCTATGAAGATGAGG	CGATCCTTCCACTTCTCAGCTA
FST	69	AAAGCACTCCGGATCTTGC	AGGAAAGCTGTAGTCCTGGTCTT
FZD2	1	GGTGTCGGTGGCCTACAT	GAGAAGCGCTCGTTGCAC
FZD5	64	CCCAGCCTGTCGCTAAACT	GGATTCCAGGGAAAGGACTC
GAPDH	45	TCCACTGGCGTCTTCACC	GGCAGAGAGATGACCCTTTT
GJA1	88	GCCTGAACTTGCCTTTTCAT	CTCCAGTCACCCATGTTGC
IGFBP6	52	ACCATCGAGGCTTCTACCG	CACACCAGCAGGGACCTC
IGFBP7	51	CTGTCCTCATCTGGAACAAGG	TGAATGGCCAGGTTGTCC
LRP5	32	GAACCTGCTGACCTGTGGAG	CTGTGGCACATGCAAACTG
MMP3	36	CAAAACATATTTCTTTGTAGAGGACAA	TTCAGCTATTTGCTTGGGAAA
MT1X	15	CTTCTCCTTGCCTCGAAATG	AGGCACAGGAGCCAACAG
NODAL	52	GGCGAGTGTCCTAATCCTGT	GCTGGTAACGTTTCAGCAGACT
PITX2	89	CTCCTGAGAGCCGAAAAGAG	CCCCTGCTGGCTTTTATCTT
POSTN	41	GAACCAAAAATTAAAGTGATTGAAGG	TGACTTTTGTTAGTGTGGGTCCT
PRG4	87	AAGAACTGGCCTGAATCTGTGT	CAGGGCACTTCTGTACAGGTT
ROR2	17	CCCCTCATTAACCAGCACAA	TTCCCAAACCGGTCCTCT
SFRP4	88	GCCTGAAGCCATCGTCAC	CCATCATGTCTGGTGTGATGT
SPARC	85	ACCCGCTTTTTCGAGACC	CAAGATCCTTGTCGATATCCTTC
TGFBI	19	TGGTCCATGTCATCACCAAT	TGCAAGTTCATCCCCTCTTT
THBS4	57	CTACCGCTGGTTCCTACAGC	GAGCCTTCATAAAATCGTACCC
TNMD	35	GATTAAAGTGATTCCTGAATTTTCTGA	AAGTTGTGGTAATTTCTTCATTCTCA
WISP2	13	CACCTCCTGGCCTTCTCC	GGGGCAGGTACATGGTGT
WNT5A	71	TGGTGCCTGATATCTCAAAGTC	GAGAAATAACCCCAGAGTAAACTGTAA

Table 1: Universal Probe Library number and sequences of the forward and reverse primers for LightCycler real-time RT-PCR of the selected 30 genes

4. Results

4.1. Selection of differentially expressed genes on Agilent microarrays

Starting with 41'000 oligos present on each Agilent array, about 6'000 showed a significant (p-value < 0.01) up- and downregulation in both tumor pools compared to the reference tissue pool, whereas roughly only half of them distinguished the two tumor pools from each other (*Figure 10*). Thus the two tumors showed less differences in gene expression than the two tumors individually compared to the reference tissue. To reduce the number of genes to be further analyzed, more stringent criteria were applied, namely, besides the mentioned p-value, a fold-change of at least two, an absolute expression level of more than 500, and the selection of only known genes. This considerably reduced the numbers of genes in each comparison (*Figure 10*).

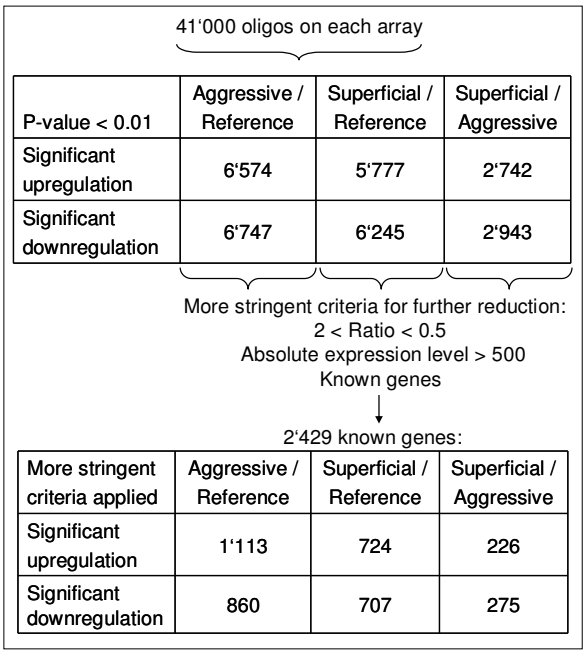

Figure 10: Processing of the array data to select for the most significant differentially expressed known genes

4.2. Verification of microarray results using real-time RT-PCR

To verify microarray results, the differential expression of the following genes was analyzed using real-time RT-PCR (*Table 2*). Their selection based on the criteria biological function, ratio and absolute expression level. Some biologically interesting genes were selected, although their ratios and absolute expression levels were rather low. This allowed to judge the ratio and absolute expression level, over which a gene trustworthily can be considered as differentially expressed.

Biological function	***Genes***
Wnt signalling	APCDD1, CDC73, DKK3, FGF18, FRZB, FZD2, FZD5, GJA1, LRP5, ROR2, SFRP4, WISP2
TGFβ signalling	FST, NODAL, PITX2, SPARC, TGFBI
PI3K-AKT signalling	IGFBP6, IGFBP7
Extracellular matrix associated	ASPN, DCD, MMP3, POSTN, PRG4, SPARC, THBS4, TNMD
Angiogenesis	ANGTPL7, CTHRC1

Table 2: Selected genes for real-time RT-PCR analysis

The differential expression of all 30 genes tested could be confirmed. Both the pooled RNA samples that were used for the microarray experiments, as well as each individual tumor sample alone needed to build up these pools were analyzed. As a representative example, *Figure 11* depicts the results obtained for the Wnt-signalling ligand WNT5A. It is highly overexpressed in aggressive fibromatoses compared to superficial fibromatoses and reference fibrous tissues, as seen in the microarray studies where pooled samples were analyzed (*a*), as well as in the real-time RT-PCR experiments, where additionally the expression in each individual sample alone was measured (*b*).

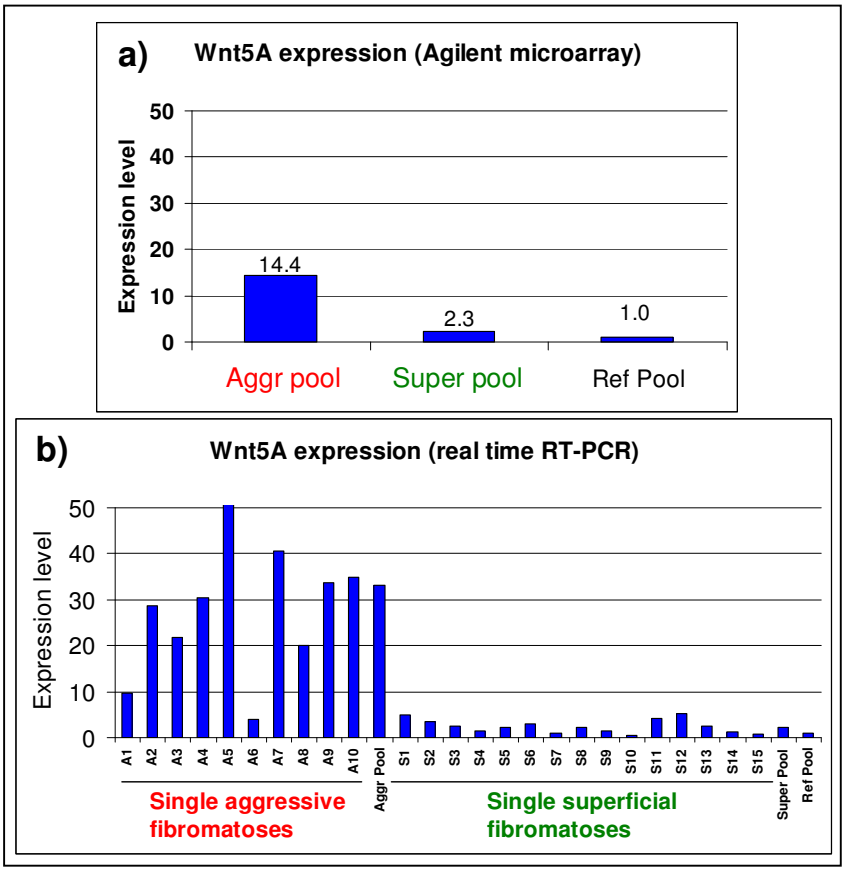

Figure 11: Expression of WNT5A in aggressive and superficial fibromatoses and reference fibrous tissues as measured using Agilent microarrays (*a*) and real-time RT-PCR (*b*). Aggr pool: aggressive fibromatosis RNA pool, Super pool: superficial fibromatosis RNA pool, Ref pool: reference fibrous tissue RNA pool.

4.3. Hierarchical clustering of the selected genes

Using the Rosetta Resolver software, the logarithmus of the ratio of tumor (both pools and individual samples) to reference fibrous tissue (pool) for the selected 2'429 genes were hierarchically clustered. This approach allows to detect similarities in the profile of selected differentially expressed genes (y-axis) in individual samples (x-axis) (*Figure 12*). The color red indicates that the selected gene is overexpressed in the corresponding tumor tissue as compared to the reference fibrous tissue, whereas the color green signifies the reverse situation. Black corresponds to no differential expression of the selected gene between the tumor and the reference fibrous tissue.

The similarities in the gene expression profiles between the different tumors are exemplified by the bar-tree on top of the figure, connecting the tumors with each other. The lower the level of the horizontal bar that connects two tumors or groups of tumors with each other, the higher is the similarity of their gene expression profiles, and vice versa. Thus the gene expression profile of the superficial fibromatosis pool (Super pool) is most similar to the one of the single individually analyzed superficial fibromatosis (Super2). On the other hand, the two samples of superficial fibromatoses show the highest divergence in their expression profiles in comparison to the six samples of aggressive fibromatoses. Therefore, this clustering analysis clearly indicates that the samples of the two tumors can be separated from each other by comparing their expression profiles for the 2'429 selected genes.

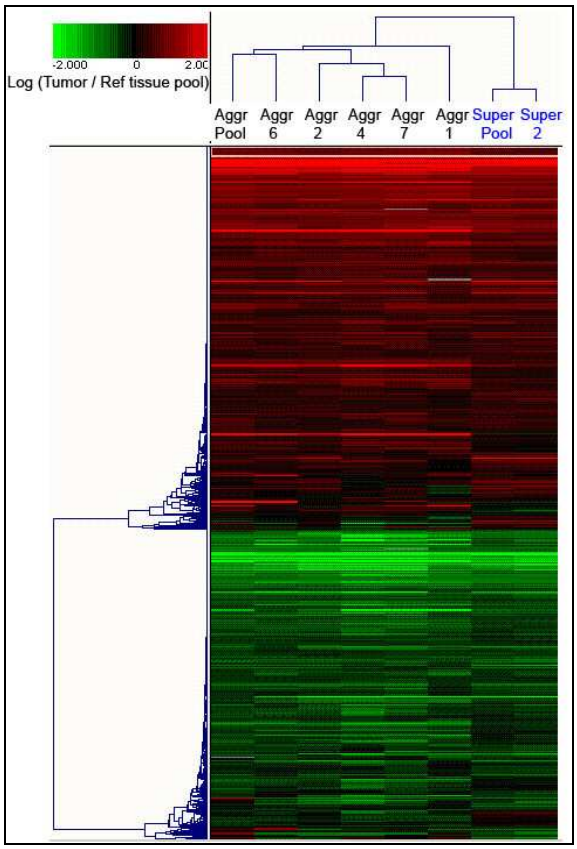

Figure 12: Hierarchical clustering of the 2'429 selected genes

4.4. Functional annotation of the selected genes

For functional annotation of the selected 2'429 genes, a biostatistical analysis was performed using the database DAVID (Database for Annotation, Visualization and Integrated Discovery). DAVID generates chart lists of biological processes that are statistically overrepresented in the uploaded list of genes. Thus the biological processes in the chart list that have a p-value of less then 0.01 are those processes that have more representatives in the uploaded gene list then expected by random selection, and therefore most likely differentiate the tissues being analyzed from each other.

4.4.1. Biological processes commonly differentiating the two tumors from the reference fibrous tissue

Figure 13 depicts the biological processes being overrepresented in the list of genes commonly differentiating the two fibromatoses from the reference fibrous tissues.

Chart list of biological processes commonly differentiating the two tumors from the reference fibrous tissues

Biological process	*# genes*	*p-value*
ECM-receptor interaction	52	2.81E-04
Focal adhesion	48	0.001
p53 signaling pathway	8	0.001
One carbon pool by folate	11	0.001
Glycolysis / Gluconeogenesis	9	0.001
Insulin signaling pathway	10	0.001
TGF-β-signaling pathway	17	0.003
Fatty acid metabolism	12	0.004
Cell cycle	29	0.004
Glycerolipid metabolism	14	0.005
mTOR signaling pathway	7	0.005
Glycerophospholipid metabolism	11	0.005
Colorectal cancer	10	0.006
ErbB signaling pathway	7	0.006
Wnt signaling pathway	12	0.007
Starch and sucrose metabolism	7	0.007
Complement and coagulation cascades	6	0.008
Glycan structures - biosynthesis 1	5	0.008
Adherens junction	17	0.008
Regulation of actin cytoskeleton	13	0.009
Cell adhesion molecules (CAMs)	9	0.010

Figure 13: DAVID-generated chart list of biological processes significantly (p-value < 0.01) overrepresented among the uploaded list of genes commonly differentiating the two tumors from the reference fibrous tissues. In the column "# genes" are the numbers of genes listed that belong to the corresponding biological processes. The color codes are explained in the text.

Due to a personal rating of the biological significance of the represented processes in context of the pathogenesis of fibromatoses and the fusions of certain processes including the same group of genes, some adaptations were performed in this chart list.

Both the processes 'Wnt signalling pathway' and 'Colorectal cancer' (color code blue) contain genes involved in the Wnt signalling pathway and were therefore fused in the single process 'Wnt signalling pathway'. In the adapted list of biological processes (*Figure 14*), it is ranked at position 1, due to its crucial role in the pathogenesis of fibromatoses (*Section 2.2.1.*). The TGFβ signalling pathway (color code violet) was

moved at position 2, because of its impact on the formation of superficial fibromatoses (*Section 2.2.2.*). The processes categorized using the color code orange (p53 signalling pathway, Glycolysis/Gluconeogenesis, Insulin signalling pathway, mTOR signalling pathway, ErbB signalling pathway) have one feature in common: the PI3K-AKT pathway (*Section 1.3.*). Therefore, their genes were summarized in one process and named after this pathway that is mainly unknown so far concerning a contribution to the pathogenesis of fibromatoses. It was set at position 3. The extracellular matrix components present among the genes belonging to the two processes standing up most in the list (color code pink: ECM-receptor interaction, Focal adhesion) were fused in the separate new process 'Extracellular matrix (ECM)' at position 4, since the DAVID-based functional categorization does not contain the separate process 'ECM'. The prominent appearance of ECM components in histological sections of fibromatoses has been pointed at in *Section 2.1.* All genes listed among the two pink processes were unified in the process 'ECM-receptor interaction' at position 5. These genes play a central role in the regulation of migration and invasion of tumor cells. The same holds true for the two processes in red (Adherens junction, Cell adhesion molecules (CAMs)) at the bottom of the list. Their genes were joined under the process name 'Adherens junction and cell adhesion molecules (CAMs)' at position 6. The process 'Cell cycle' in dark green, fundamental in context of tumorigenesis, was renamed into the more general term 'Proliferation' and set at position 7. The process 'Regulation of actin cytoskeleton' (color code brown) represents an important process in the pathogenesis of fibromatoses, since tumor cells show features of myofibroblasts, expressing bundles of smooth muscle actin (α-SMA) microfilaments similar to those found in smooth muscle cells (*Section 2.1.*). It was renamed into 'Cytoskeleton' and listed at position 8. The process 'complement and coagulation cascades' (light green) was ordered under the same term at position 9. In addition, the category 'Others' at position 10 includes all those selected differentially expressed genes that could not be assigned to a biological process but were characterized by high differential expressions and/or high absolute expression levels. The processes listed in *Figure 13* in grey (One carbon pool by folate, Fatty acid metabolism, Glycerolipid metabolism, Starch and sucrose metabolism, Glycan structures – biosynthesis 1) are all in context of metabolism/anabolism, known to be specifically enhanced in tumors. Since our focus of interest was laid on the processes mentioned before, they were excluded from *Figure 14*.

The numbers of genes belonging to those ten biological processes were expanded by the manual addition of differentially expressed genes that were not recognized by the automated DAVID-based functional annotation.

Adapted chart list of biological processes commonly differentiating the two tumors from the reference fibrous tissues

	Biological process	*# of genes in the tumors*	
		upregulated	*downregulated*
1)	**Wnt signalling pathway**	18	6
2)	**TGFβ signaling pathway**	15	8
3)	**PI3K-AKT signalling pathway**	5	10
4)	**Extracellular matrix (ECM)**	53	14
5)	**ECM-receptor interaction**	46	7
6)	**Adherens junction and cell adhesion molecules (CAMs)**	20	14
7)	**Proliferation**	58	59
8)	**Cytoskeleton**	21	5
9)	**Complement and coagulation cascades**	5	10
10)	**Others**	23	22

Figure 14: Adapted list of biological processes significantly overrepresented among the uploaded list of genes commonly differentiating the two tumors from the reference fibrous tissues. The numbers of genes being commonly upregulated and downregulated in the two tumors as compared to the reference fibrous tissues are indicated for each biological process. The color codes are explained in the text.

Comprehensive informations about all differentially expressed genes (name, ratio, p-value, absolute expressions) categorized into the ten biological processes can be found in *Gene list A* in the *Appendix*.

4.4.2. Biological processes differentiating the two tumors from each other

Figure 15 depicts the biological processes being overrepresented in the list of genes differentiating the two tumors from each other.

Chart list of biological processes differentiating the two tumors from each other

Biological process	*# genes*	*p-value*
Focal adhesion	28	2.53E-05
ECM-receptor interaction	27	6.43E-05
Complement and coagulation cascades	10	1.59E-04
Wnt signaling pathway	11	0.001
Colorectal cancer	7	0.003
Regulation of actin cytoskeleton	12	0.004
TGFβ-signaling pathway	7	0.007
Cell adhesion molecules (CAMs)	5	0.009

Figure 15: DAVID-generated chart list of biological processes significantly (p-value < 0.01) overrepresented among the uploaded list of genes differentiating the two tumors from each other. In the column "# genes" are the numbers of genes listed that belong to the corresponding biological processes. The color codes are explained in the text in Section 4.4.1.

A comparison of the colors representing the biological processes in *Figure 13* and *Figure 15* demonstrates that all biological processes that are significantly overrepresented in the list of genes differentiating the two tumors from each other (*Figure 15*), are also significantly overrepresented in the list of genes commonly differentiating the two tumors from the reference fibrous tissue (*Figure 13*). On the other hand, unique among the processes commonly differentiating the two tumors from the reference fibrous tissue (*Figure 13*) are the two processes PI3K-AKT signalling pathway and proliferation. This indicates that most differentially expressed genes belonging to these two processes and differentiating aggressive fibromatosis from reference fibrous tissue are identical to the ones distinguishing superficial fibromatosis from reference fibrous tissue. In addition, it suggests that those genes

must be regulated in a quantitatively (concerning ratio value) similar way in both tumors.

The chart list in *Figure 15* was adapted according to approach described in *Section 4.4.1.* Afterwards, the numbers of genes belonging to those processes were expanded by manual addition. The resulting adapted chart list is depicted in *Figure 16*. To allow a direct comparison with *Figure 14*, *Figure 16* includes the processes PI3K-AKT signalling pathway and proliferation as well, although they are not statistically overrepresented in the list of genes differentiating the two tumors from each other.

Adapted chart list of biological processes differentiating the two tumors from each other

	Biological process	***# of genes***	
		Super > Aggr	***Super < Aggr***
1)	**Wnt signalling pathway**	8	21
2)	**TGFβ signaling pathway**	10	9
3)	**PI3K-AKT signalling pathway**	6	3
4)	**Extracellular matrix (ECM)**	22	17
5)	**ECM-receptor interaction**	20	16
6)	**Adherens junction and cell adhesion molecules (CAMs)**	10	6
7)	**Proliferation**	31	25
8)	**Cytoskeleton**	14	19
9)	**Complement and coagulation cascades**	15	7
10)	**Others**	14	24

Figure 16: Adapted list of biological processes significantly overrepresented among the uploaded list of genes differentiating the two tumors from each other. The numbers of genes being upregulated in their expressions in superficial fibromatosis as compared to aggressive fibromatosis (Super > Aggr) and vice versa (Super < Aggr) are indicated for each biological process. The color codes are explained in the text in Section 4.4.1.

Comprehensive informations about all differentially expressed genes (name, ratio, p-value, absolute expressions), categorized according to the biological processes, can be obtained from *Gene list B* in the *Appendix*.

4.4.3. Single genes differentiating the two tumors from each other

Within *Gene list B* in the *Appendix*, those genes where selected that outstandingly differentiate the two tumors from each other by a high ratio and a highly significant p-value. 16 candidate genes that fulfil these selection criteria are presented in *Figure 17*. The proteins encoded by those genes represent attractive candidates for marker proteins differentiating the two tumors using immunohistochemical analysis.

a) *Upregulated in superficial fibromatosis*

				Absolute expression	
Gene	Name	Ratio	p-value	Super	Aggr
ANGPTL7	Angiopoietin-like 7	66.9	0	12779	192
TNMD	Tenomodulin	24.0	0	5597	233
DCD	Dermcidin	22.7	0	12859	566
PRG4	Proteoglycan 4	18.7	0	4629	246
DMRT2	Doublesex and mab-3 related transcription factor 2	12.3	0	1886	154
MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response	10.5	0	3158	300
NOV	Nephroblastoma overexpressed gene	8.9	0	9069	1018
THBS4	Thrombospondin 4	8.0	2.8026E-45	67565	8263

b) *Upregulated in aggressive fibromatosis*

				Absolute expression	
Gene	Name	Ratio	p-value	Aggr	Super
FGA	Fibrinogen, A alpha polypeptide	29.0	2.51E-15	1879	94
ACTN2	Actinin, alpha 2	18.0	0	1129	64
AREG	Amphiregulin (schwannoma-derived growth factor)	14.7	0	8751	598
APCDD1	Adenomatosis polyposis coli down-regulated 1	13.2	0	26641	2023
TTN	Titin	9.2	0	5824	633
PITX2	Paired-like homeodomain transcription factor 2	8.4	0	3142	373
GREM1	Gremlin 1 homolog, cysteine knot superfamily	7.1	9.75E-30	5990	823
WNT5A	Wingless-type MMTV integration site family, member 5A	7.0	0	5937	849

Figure 17: Single genes outstandingly differentiating the two tumors from each other. a) 8 genes upregulated in superficial fibromatosis, b) 8 genes upregulated in aggressive fibromatosis. The color code categorizes the genes into the following biological processes: dark green = proliferation, pink = extracellular matrix (ECM), black = others, brown = cytoskeleton, violet = TGFβ signalling pathway, light green = complement and coagulation cascades, orange = PI3K-AKT signalling pathway, blue = Wnt signalling pathway. The genes are ordered according to the ratio tumor vs. tumor.

ANGPTL7, TNMD and DCD are coding for secreted proteins known to induce proliferation of certain cell types. PRG4 and NOV are components of the extracellular matrix (ECM). DMRT2 encodes a transcription factor of uncertain function. MYOC, ACTN2 and TTN are components of the cytoskeleton. The ECM component THBS4 and the transcription factor PITX2 are involved in the TGFβ signalling pathway. FGA is a major component in the process of blood coagulation. AREG is a member of the epidermal growth factor (EGF) family known to activate the PI3K-AKT signalling pathway. The Wnt-ligand WNT5A activates the Wnt signalling pathway, whereas GREM1 and APCDD1 represent two target genes of this pathway.

4.5. Detailed analysis of signalling pathways

Since besides the Wnt, the TGFβ and the PI3K-AKT pathway, no other signalling pathway proved to be significantly overrepresented among the lists of differentially expressed genes, the following detailed analyses in this section will focus on these three pathways.

The individual differentially expressed components within each pathway were grouped according to their functions into pathway activity inducers, inhibitors and pathway target genes. This approach aimed to deduce informations about differential activities within these pathways between the tissues analyzed. The restrictions of this approach are discussed in *Section 5.4.*

The presented data are based on the analysis of pooled tissue samples.

4.5.1. The canonical Wnt signalling pathway

The components of the canonical Wnt signalling pathway were introduced in detail in *Section 1.1.*, whereas its involvement in the pathogenesis of fibromatoses was explained in *Section 2.2.1.*

- Aggressive fibromatosis vs. reference fibrous tissue

Figure 18 depicts the components of the canonical Wnt signalling pathway being differentially expressed between aggressive fibromatosis and reference fibrous tissue.

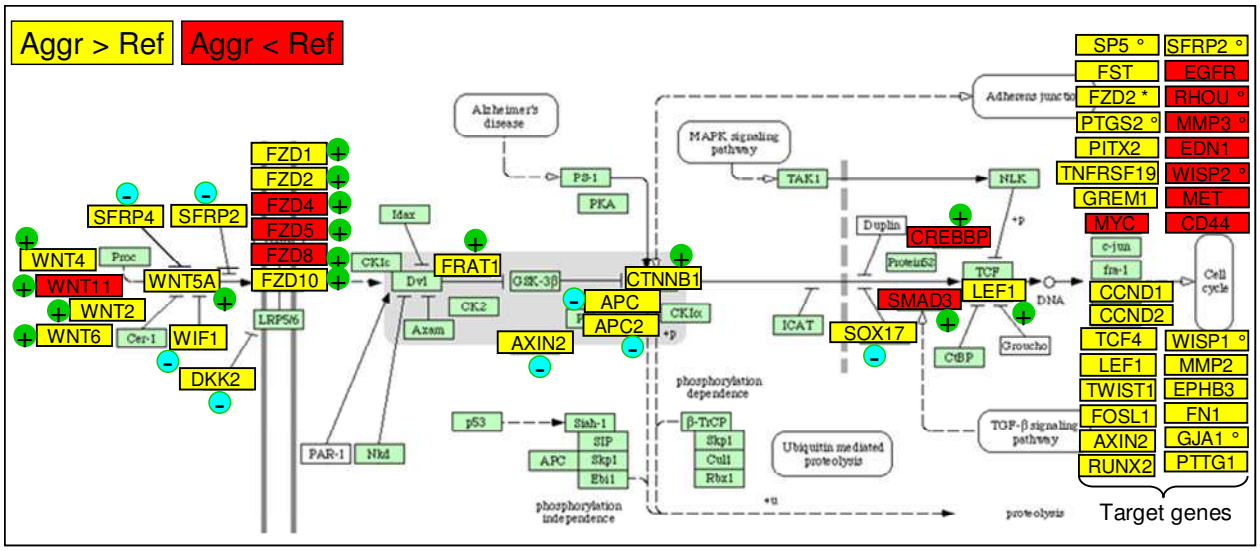

Figure 18: Between aggressive fibromatosis (Aggr) and reference fibrous tissue (Ref) differentially expressed genes of the Wnt signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor.
(+) pathway activity inducer, (-) pathway activity inhibitor, (*) FZD2 is repressed in its expression by an active Wnt pathway, (°) target genes that have not been established in human tissues, yet.

In the tumor tissue, more pathway activity inducers are overexpressed (10) than in the reference tissue (6). On the other hand, 8 pathway activity inhibitors are upregulated in their expression in the tumor, whereas no inhibitors at all could be

found to be overexpressed in the reference tissue. This could reflect the establishment of a negative feedback loop. The number of differentially expressed target genes reflecting an increased activity of this pathway in the tumor (21) is clearly higher than in the reference fibrous tissue (9).

- Superficial fibromatosis vs. reference fibrous tissue

Figure 19 shows the same approach, but applied on the comparison between superficial fibromatosis and reference fibrous tissue.

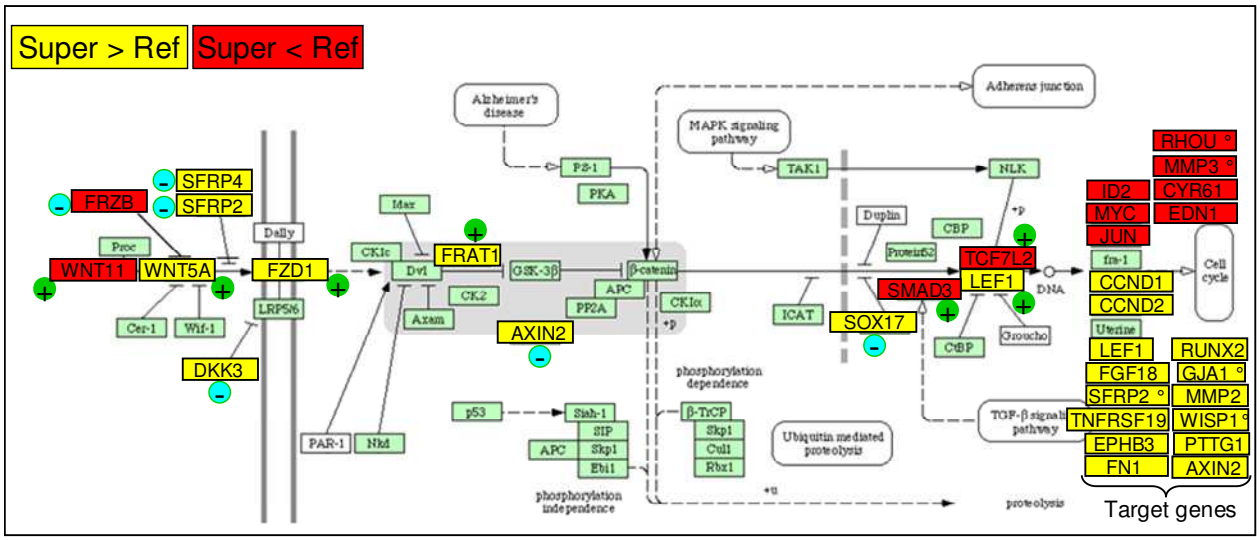

Figure 19: Between superficial fibromatosis (Super) and reference fibrous tissue (Ref) differentially expressed genes of the Wnt signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor.
(+) pathway activity inducer, (-) pathway activity inhibitor, (°) target genes that have not been established in human tissues, yet.

In superficial fibromatosis and reference tissue, similar numbers of pathway inducers are overexpressed (4 and 3, respectively), whereas more inhibitors are upregulated in the tumor (5 vs. 1). Again, the overexpressed inhibitors may indicate a negative feedback loop. An analysis of differentially expressed target genes reveals that more of them are upregulated in the tumor tissue (14) as compared to the reference fibrous tissue (7), suggesting an increased activity of this pathway in superficial fibromatosis as compared to the reference tissue.

- Aggressive and superficial fibromatosis vs. reference fibrous tissue

The analysis of canonical Wnt pathway components commonly deregulated between the two tumors and the reference fibrous tissue is summarized in *Figure 20*.

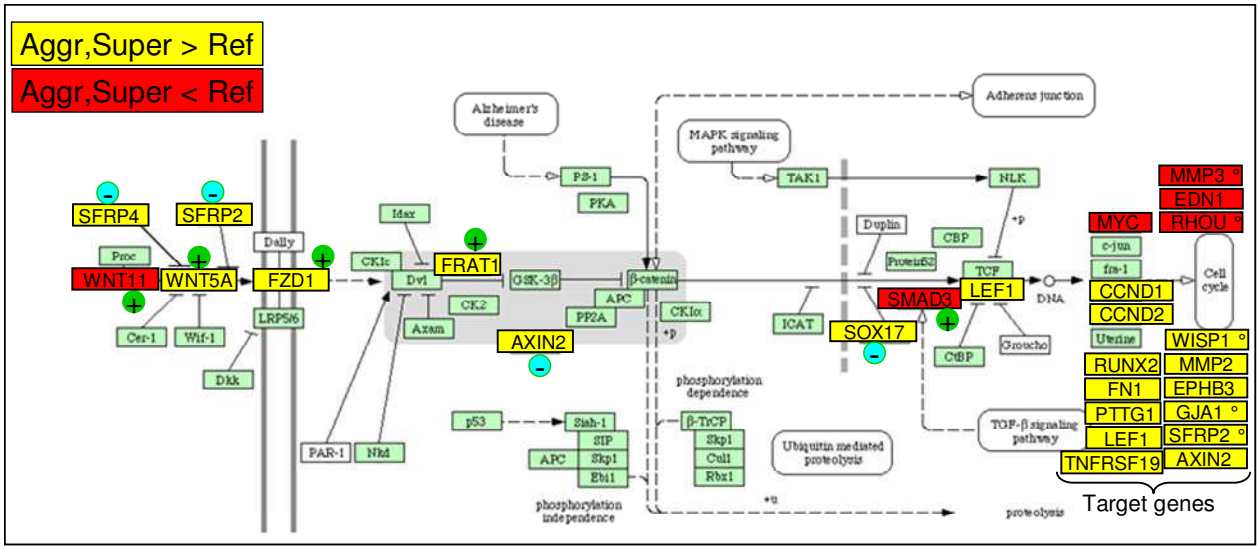

Figure 20: Genes of the Wnt signalling pathway commonly differentiating aggressive and superficial fibromatoses from reference fibrous tissue. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in both tumors.
(+) pathway activity inducer, (-) pathway activity inhibitor, (°) target genes that have not been established in human tissues, yet.

48% (24/50) of the pathway components found to be deregulated in aggressive fibromatosis are also deregulated in superficial fibromatosis. On the other hand, even 77% (24/31) of the differentially expressed genes detected in superficial fibromatosis behave in the same way in aggressive fibromatosis. This reflects prominent similarities in the deregulation of the Wnt signalling pathway in both aggressive and superficial fibromatosis.

- Hierarchical clustering of the canonical Wnt pathway target genes

Figure 21 depicts a hierarchical clustering of the 35 canonical Wnt signalling pathway target genes found to be differentially expressed between the three tissue pools. All gene expression arrayed samples, also the six individually analyzed tumors (Aggr1, Aggr2, Aggr4, Aggr6, Aggr7, Super2), were included in this clustering. The dominant color red indicates that the majority of the target genes are overexpressed in the tumors in comparison to the reference fibrous tissue. Whereas in *Figure 12*, the analysis of all 2'429 selected genes delivered two clusteres separating aggressive fibromatosis from the superficial fibromatosis samples, such a strict separation disappears when clustering just the 35 Wnt pathway target genes.

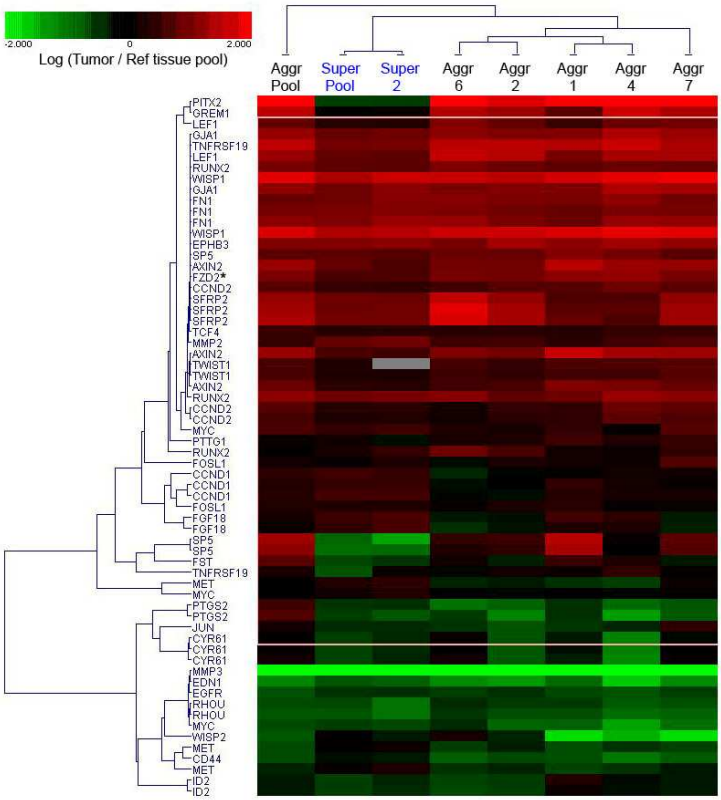

Figure 21: Hierarchical clustering of canonical Wnt target genes differentially expressed between the three tissue pools

4.5.2. The TGFβ signalling pathway

The components of the TGFβ signalling pathway were introduced in detail in *Section 1.2.*, whereas its involvement in the pathogenesis of superficial fibromatosis was explained in *Section 2.2.2.*

- Aggressive fibromatosis vs. reference fibrous tissue

Figure 22 depicts the components of the TGFβ signalling pathway being differentially expressed between aggressive fibromatosis and reference fibrous tissue.

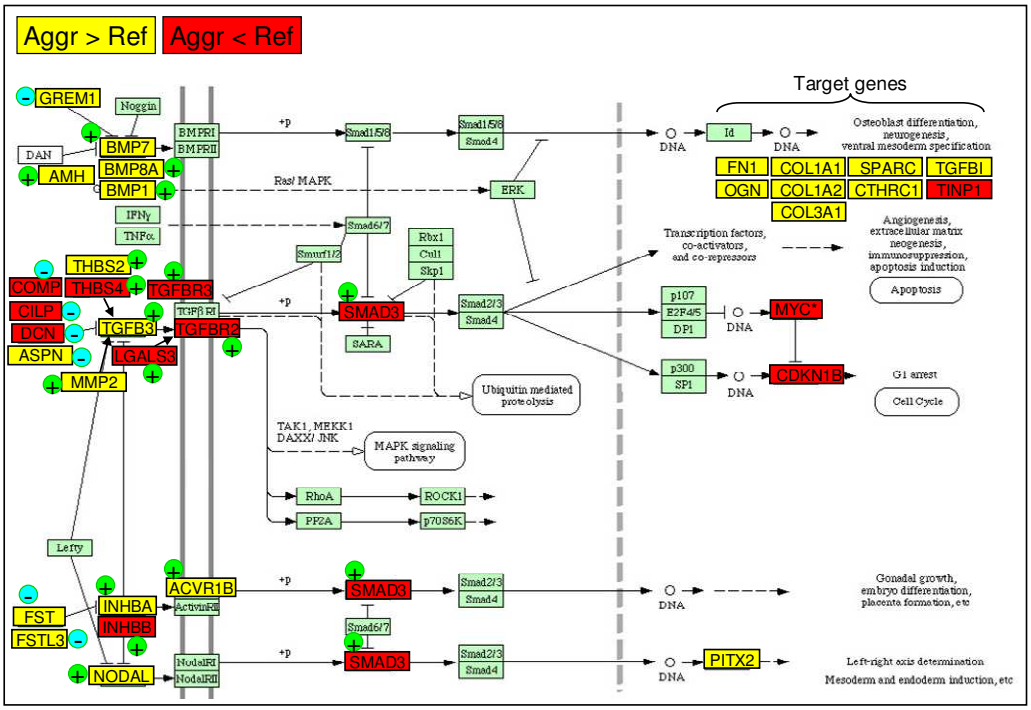

Figure 22: Between aggressive fibromatosis (Aggr) and reference fibrous tissue (Ref) differentially expressed genes of the TGFβ signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor.
(+) pathway activity inducer, (-) pathway activity inhibitor, (*) MYC is repressed in its expression by an active TGFβ signalling pathway.

More pathway activity inducers are overexpressed in aggressive fibromatosis (10) than vice versa (6). In the tumor and the reference fibrous tissue, similar numbers of pathway inhibitors (4 vs. 3) are upregulated. The number of differentially expressed TGFβ signalling target genes reflecting an increased activity of this pathway is higher in the tumor (10) than in the reference tissue (2).

- Superficial fibromatosis vs. reference fibrous tissue

Figure 23 presents the TGFβ pathway components that are transcriptionally deregulated between superficial fibromatosis and reference fibrous tissue.

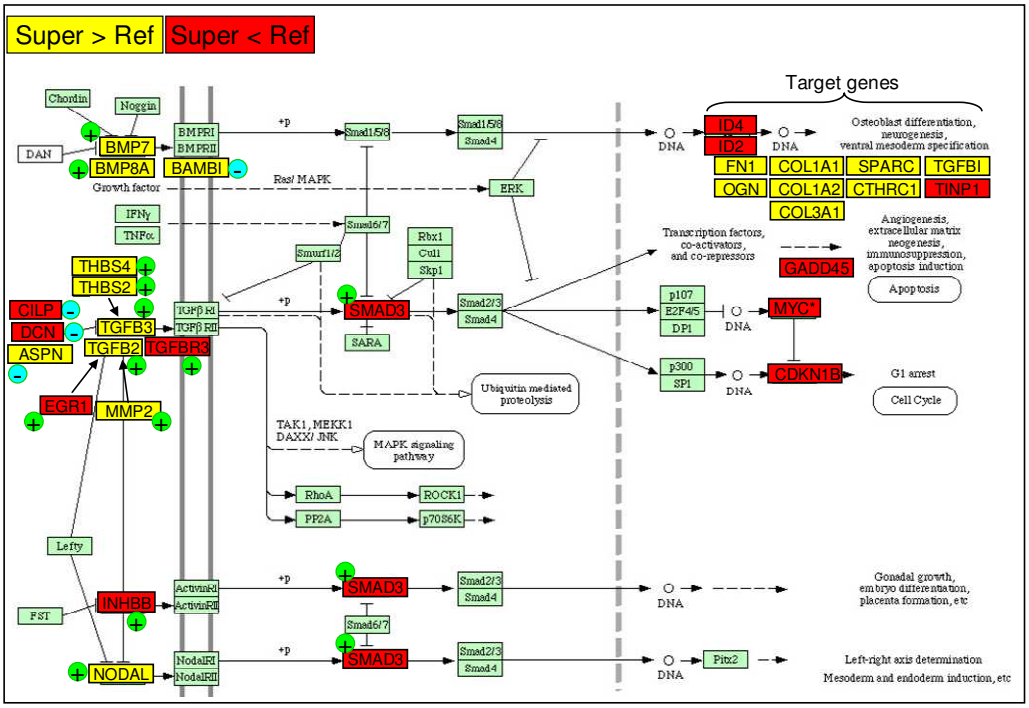

Figure 23: Between superficial fibromatosis (Super) and reference fibrous tissue (Ref) differentially expressed genes of the TGFβ signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor.
(+) pathway activity inducer, (-) pathway activity inhibitor, (*) MYC is repressed in its expression by an active TGFβ signalling pathway.

Among the differentially expressed pathway activity inducers, 8 are upregulated in superficial fibromatosis, whereas only 4 of them in the reference fibrous tissue. In both tissues, an equal number of pathway inhibitors (2) are overexpressed. The number of differentially expressed TGFβ signalling target genes reflecting an increased activity of this pathway in the tumor (9) is higher than in the reference tissue (5).

- Aggressive and superficial fibromatosis vs. reference fibrous tissue

Between both tumors and the reference fibrous tissue commonly deregulated components of the TGFβ pathway are presented in *Figure 24*.

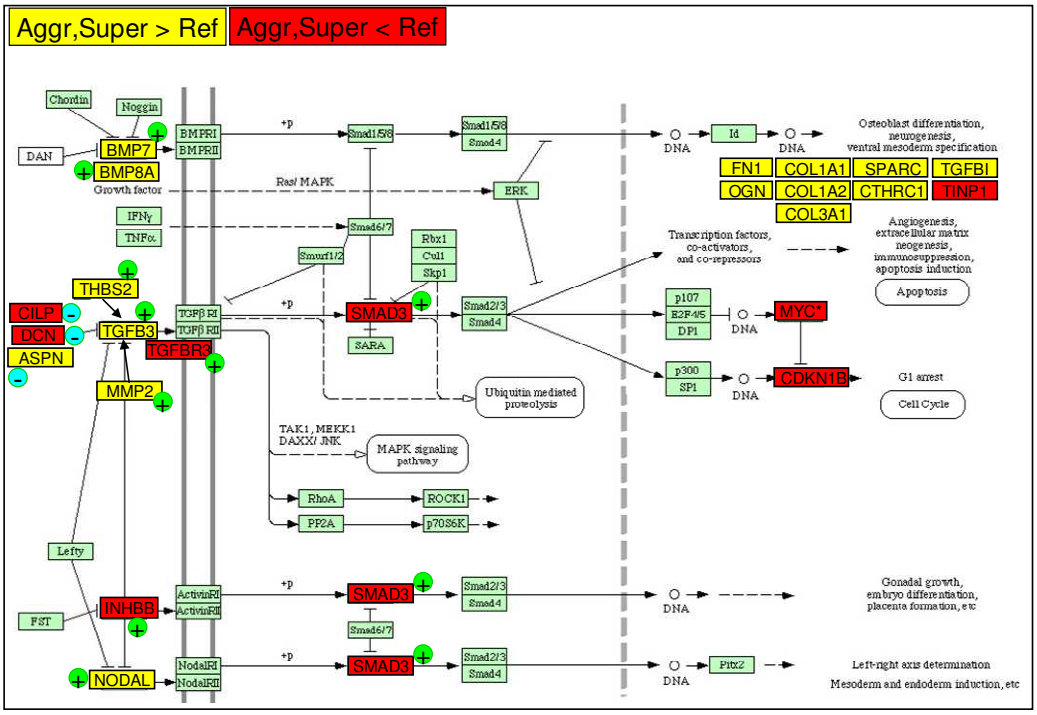

Figure 24: Genes of the TGFβ signalling pathway commonly differentiating aggressive and superficial fibromatoses from reference fibrous tissue. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in both tumors.
(+) pathway activity inducer, (-) pathway activity inhibitor, (*) MYC is repressed in its expression by an active TGFβ signalling pathway.

66% (23/35) of the genes found to be deregulated in aggressive fibromatosis are accordingly deregulated in superficial fibromatosis. And even 77% (23/30) of the differentially expressed genes detected in superficial fibromatosis behave in the same way in aggressive fibromatoses. This reflects prominent similarities in the deregulation the TGFβ signalling pathway in both aggressive and superficial fibromatosis.

4.5.3. The PI3K-AKT signalling pathway

The components of the PI3K-AKT signalling pathway were introduced in detail in S*ection 1.3.* There, it is explained that the central component of this pathway, AKT, is not a transcription factor, but a serine/threonine-kinase modulating the activities of a plethora of downstream targets on the protein level by phosphorylation. These proteins in turn transmit the signal on the protein level to other target proteins or regulate the transcription of target genes (in the case of transcription factors), resulting in an increasing branching and expansion of the downstream signalling pathways. There exists no defined, central transcription factor in the PI3K-AKT pathway, as it is the case in the Wnt (TCF) or the TGFβ (Smad) signalling pathways, but a crosstalk to a complex network of individual signal transduction routes. Therefore, informations about differential activities in the PI3K-AKT pathway between different tissues can not be deduced by simply enumerating a limited number of differentially expressed target genes. For this reason, the examination of the pathway activity was restricted in this study to the enumeration of differentially expressed pathway activity inducers and inhibitors upstream of AKT. Downstream of AKT, the focus was laid on the analysis of the biological functions of the differentially expressed pathway components in terms of a possible contribution of these genes to the pathogenesis of fibromatoses.

- Aggressive fibromatosis vs. reference fibrous tissue

Figure 25 depicts the components of the PI3K-AKT pathway that are differentially expressed between aggressive fibromatosis and reference fibrous tissue.

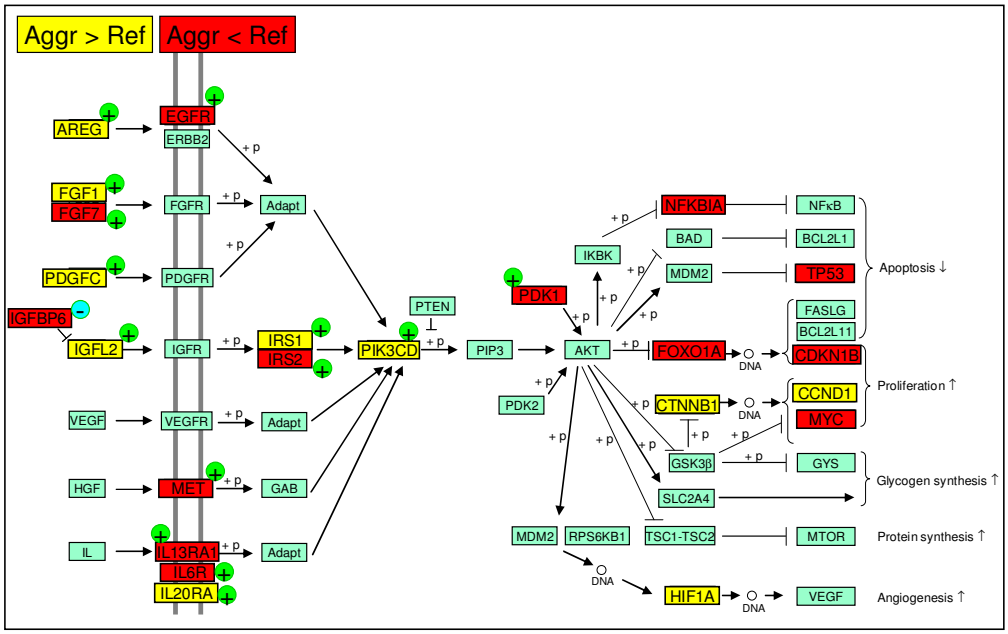

Figure 25: Between aggressive fibromatosis (Aggr) and reference fibrous tissue (Ref) differentially expressed genes of the PI3K-AKT signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor.
(+) pathway activity inducer, (-) pathway activity inhibitor.

Upstream of AKT, seven genes responsible for an activation of this pathway are upregulated in their expression in the tumor, whereas an equal number is overexpressed in the reference tissue. Just one inhibitor, IGFBP6, is downregulated

in the tumor as compared to the reference fibrous tissue. Therefore, a differential activation of the PI3K-AKT pathway in aggressive fibromatosis can not be deduced.

Downstream of AKT, three components known for their apoptosis-inducing activity are downregulated in aggressive fibromatosis as compared to the reference fibrous tissue, namely an inhibitor of NFκB (NFKBIA), the transcription factor forkhead box O1A (FOXO1A) and the tumor protein p53 (TP53).

In addition, some genes involved in the regulation of proliferation are differentially expressed in aggressive fibromatosis. Cyclin / cyclin-dependent kinase complex inhibitor 1B (CDKN1B), an inhibitor of cell cycle progression, is downregulated, whereas cyclin D1 (CCND1) and β-catenin, two inducers of proliferation, are upregulated in the tumor. On the other hand, c-myc (MYC), another stimulator of proliferation, is downregulated in aggressive fibromatosis as compared to the reference fibrous tissue.

The major inducer of angiogenesis, hypoxia-inducible factor 1 α subunit (HIF1α), is more highly expressed in the tumor.

- Superficial fibromatosis vs. reference fibrous tissue

The differentially expressed pathway components between superficial fibromatosis and reference fibrous tissue are shown in *Figure 26*.

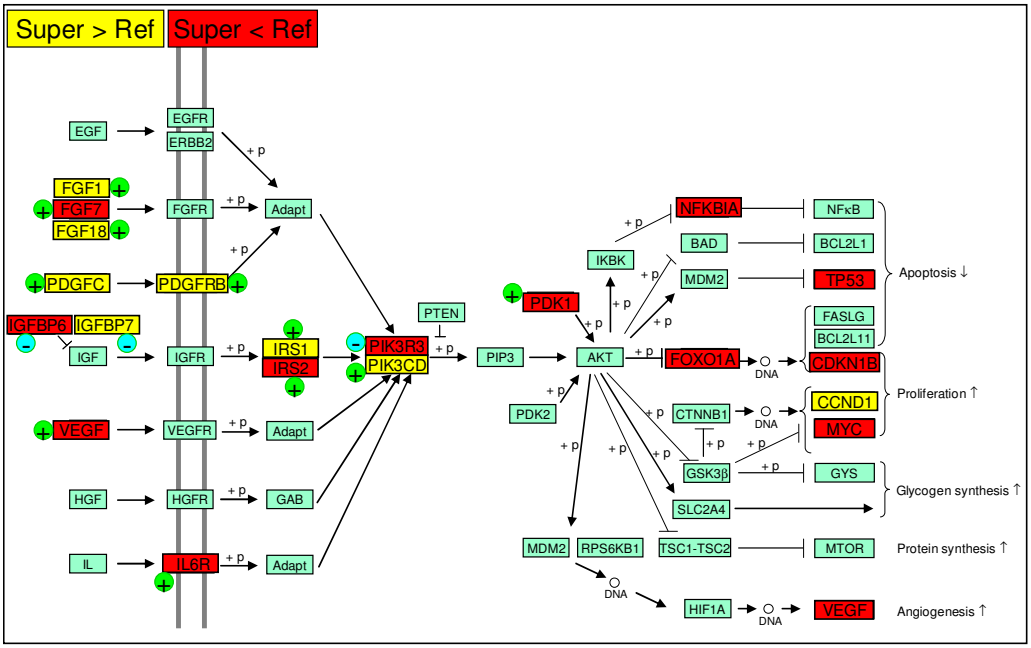

Figure 26: Between superficial fibromatosis (Super) and reference fibrous tissue (Ref) differentially expressed genes of the PI3K-AKT signalling pathway. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in the tumor. (+) pathway activity inducer, (-) pathway activity inhibitor.

Upstream of AKT, similar numbers of pathway activity inducers and inhibitors are overexpressed in the tumor (6 inducers, 1 inhibitor) and the reference fibrous tissue (5 inducers, 2 inhibitors).

Most of the genes found to be deregulated downstream of AKT in aggressive fibromatosis behave equally in superficial fibromatosis. The exceptions represent β-catenin and HIF1α that do not appear any more in the list of deregulated genes in superficial fibromatosis. Instead, the HIF1α target gene VEGF is downregulated in superficial fibromatosis as compared to the reference fibrous tissue.

- Aggressive and superficial fibromatosis vs. reference fibrous tissue

Between both tumors and the reference fibrous tissue commonly deregulated components of the PI3K-AKT pathway are presented in *Figure 27*.

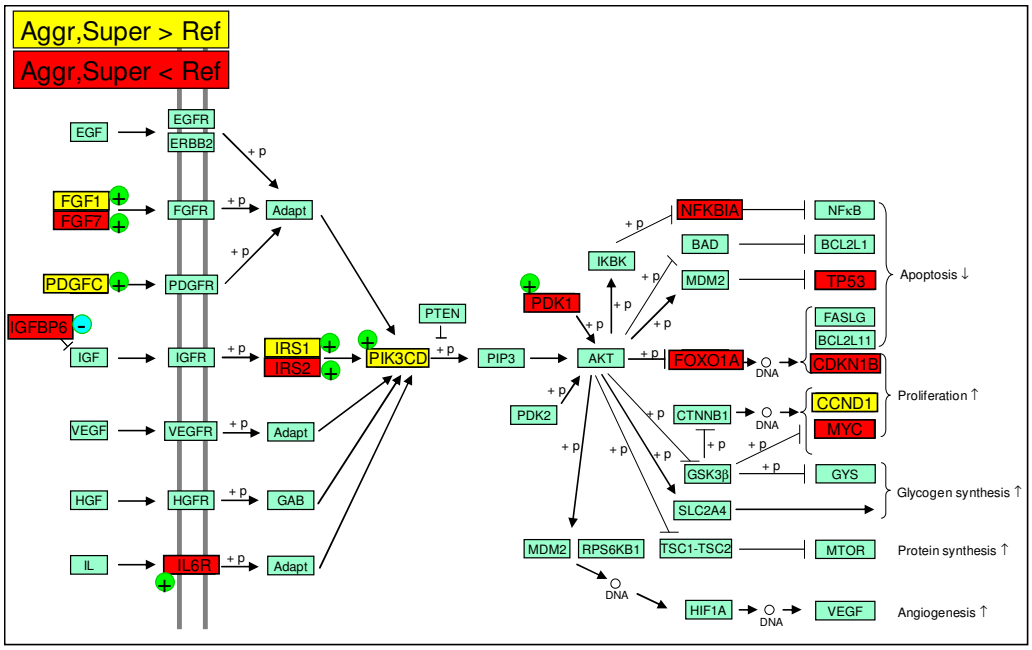

Figure 27: Genes of the PI3K-AKT signalling pathway commonly differentiating aggressive and superficial fibromatoses from reference fibrous tissue. Components represented as yellow rectangles are overexpressed, whereas those symbolized as red rectangles are downregulated in both tumors. (+) pathway activity inducer, (-) pathway activity inhibitor.

15 of the 23 (65%) PI3K-AKT components found to be deregulated in their expressions in aggressive fibromatosis were in the same direction modulated in superficial fibromatosis. On the other hand, 15 out of 21 (71%) components that proved to be differentially expressed in superficial fibromatosis behaved in the same way in aggressive fibromatosis. This demonstrates a pronounced overlap of the differentially expressed PI3K-AKT pathway components in aggressive and superficial fibromatosis.

5. Discussion

5.1. Summary of the results

Using Agilent microarray gene expression analysis, 2'429 genes could be determined differentiating aggressive and superficial fibromatoses from reference fibrous tissue or the two tumors from each other.

A hierarchical clustering analysis of those genes built two clusters, separating the samples of aggressive fibromatosis from the ones of superficial fibromatosis.

Functional annotation of selected genes commonly differentiating the two tumors from the reference fibrous tissue revealed the following biological processes to be statistically overrepresented: Wnt signalling pathway, TGFβ signalling pathway, PI3K-AKT signalling pathway, extracellular matrix (ECM), ECM-receptor interaction, adherens junction and cell adhesion molecules (CAMs), proliferation, cytoskeleton, complement and coagulation cascades.

Interestingly, with the exceptions of the PI3K-AKT signalling pathway and proliferation, all the biological processes being overrepresented in the list of genes commonly differentiating the two tumors from the reference tissue, are also significantly overrepresented in the list of genes differentiating the two tumors from each other.

The differentially expressed components of the Wnt, TGFβ and PI3K-AKT signalling pathways were analyzed in detail. Although some restrictions of the validity of the resulting statements must be considered, some interesting hints could be deduced from these analyses.

The numbers of regulated pathway activity inducers and/or pathway target genes indicated that aggressive and superficial fibromatoses are characterized by increased signalling activities in the Wnt and TGFβ pathways as compared to the reference fibrous tissue.

In addition, the overexpression of numerous Wnt pathway activity inhibitors in fibromatoses suggests that a negative feedback loop mechanism may try to counterbalance an excessive Wnt signalling pathway activity in both tumors.

Concerning the PI3K-AKT pathway upstream of AKT, the analysis of pathway inducers and inhibitors did not reveal a differential activity of this pathway between the tissues. Vast branching and expansion of the PI3K-AKT pathway downstream of AKT make an analysis of a limited number of pathway target genes impossible. Nevertheless, the analysis of the biological processes the differentially expressed components downstream of AKT are involved in revealed some findings relevant for the pathogenesis of fibromatoses. In both fibromatoses, genes inducing apoptosis were found to be downregulated, whereas differentially expressed genes regulating proliferation favour an induction of proliferation. Therefore, differentially expressed PI3K-AKT components downstream of AKT may contribute to the pathogenesis of fibromatoses.

For all three pathways, there is an extensive overlap of differentially expressed components in aggressive and superficial fibromatoses.

5.2. Comparison of own results with data published in the literature

A literature screen was performed to find genes that are reported to be differentially expressed between fibromatoses and reference fibrous tissues or – very rarely – between aggressive and superficial fibromatoses.

5.2.1. Consensus with published data

Overall, 234 genes could be gathered. The statements for 93 (40%) of them could be confirmed by our own data. Those 93 genes are summarized in the *Gene list C* in the *Appendix*. There, a link to the corresponding literature, a short description of the experiment and a comparison to the own results can also be obtained. That the differential expression of 93 out of 234 genes could be confirmed is much more than expected when comparing two randomly selected lists of differentially expressed genes: our 2'429 selected genes represent roughly only 10% of all known genes present on the Agilent microarray used. Therefore, a random selection would yield an overlap of 10% at maximum. This high agreement with literature data is even more astonishing, when thinking about the different techniques that were applied to produce these data, ranging from analyses on the mRNA level (Microarray, RT-PCR, Northern blot), through studies done on protein extracts (Western blot, 2D-gel-electrophoresis and mass-spectrometry), to experiments using cell culture supernatants (ELISA). The high consensus to the literature data therefore underlines the trustworthiness of our data.

The published results of expression analyses for 141 genes could not be confirmed by our own data. They are summarized in the *Gene list D* in the *Appendix*, including a link to the corresponding literature, a short description of the experiment and a comparison to the own results.

5.2.2. Genes belonging to nine biological processes

In *Section 4.4.1*., the functional annotation of selected genes commonly differentiating the two tumors from the reference fibrous tissue revealed that nine biological processes are statistically overrepresented (*Figure 28, Own data.*). Most of the genes reported in the literature to differentiate aggressive or superficial fibromatosis from reference fibrous tissue belong to these nine biological processes as well (*Figure 28, Literature, total*).

	Biological process	Own data	Literature							
			total	Denys	Skubitz	Pan	Qian	Lee	Satish	Zhang
1)	Wnt signalling pathway	24	13	3	2	0	1	1	0	0
2)	TGFβ signaling pathway	23	23	0	5	0	2	2	0	3
3)	PI3K-AKT signalling pathway	15	12	2	0	0	0	0	0	0
4)	Extracellular matrix (ECM)	67	56	11	16	3	7	5	3	5
5)	ECM-receptor interaction	53	28	2	11	2	3	5	1	3
6)	Adherens junction and cell adhesion molecules	34	12	2	1	2	1	0	0	2
7)	Proliferation	117	49	15	7	1	2	3	1	5
8)	Cytoskeleton	26	15	6	0	1	0	1	0	0
9)	Complement and coagulation cascades	15	5	1	0	0	0	1	0	0

Figure 28: Comparison of own results with literature data by means of the numbers of genes belonging to the nine biological processes determined in *Section 4.4.1.* Total: summary of all expression studies. Microarray expression studies are listed individually.

Of those genes, numerous are derived from microarray gene expression experiments:

- Aggressive fibromatosis

In one study (*Figure 28*, *Literature, Denys*), the gene expression of four primary cell cultures of aggressive fibromatoses (no proof of tumoral origin was provided) was compared with the one in cultures of normal fascia using Affymetrix microarrays. The authors found 69 genes to be differentially expressed (33 genes upregulated, 36 genes downregulated). The most numerously represented biological processes are the ECM (e.g. type VI collagen, proteoglycan 1, ADAM metallopeptidase domain 19 (ADAM19), matrix metalloproteinases 3 and 7 (MMP3, MMP7)) and proliferation, including cyclin D2, insulin-like growth factor 2 (IGF2), insulin-like growth factor-binding protein 6 (IGFBP6) and neurite growth-promoting factor 2 (MDK).

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In a second study (*Figure 28, Literature, Skubitz*), 12 samples of aggressive fibromatosis tissues were compared with 16 non-neoplastic tissues, including normal adipose, cervix, colon, kidney, liver and lung, using Affymetrix-based gene expression analysis. 30 genes were found to be significantly upregulated in aggressive fibromatosis, most of them belonging to the biological processes ECM / ECM-receptor interaction, proliferation and TGFβ signalling pathway. The ECM-components include type I, III, V, VI, XI, XII, XIV collagen, spondin 2 (SPON2), biglycan (BGN), cartilage oligomeric protein (COMP) and versican (CSPG2). Genes involved in the process of proliferation represent TGFβ3 (TGFB3), angiopoietin-like 2 (ANGPTL2), tumor necrosis factor ligand superfamily member 4 (TNFSF4) or stem-cell growth factor (CLEC11A). In context of TGFβ-signalling pathway, TGFβ3, asporin (ASPN), COMP, type I and type II collagen could be found.

- Superficial fibromatosis

Comparing the gene expression of 6 palmar superficial fibromatosis tissues with the one in 2 unaffected palmar fascia using Atlas microarrays (*Figure 28, Literature, Pan*), 23 genes were reported to be differentially expressed. Three of them belong to the extracellular matrix (laminin, beta 3 (LAMB3), tetranectin (CLEC3B) and amyloid A4 precursor (APP)), two to the biological process adherens junction (intercellular adhesion molecule 2 (ICAM2), erythrocyte membrane protein band 4.9 (EPB49)) and one each to the processes proliferation (tumor necrosis factor superfamily member 12 (TNFSF12)) and cytoskeleton (tubulin alpha 3 (TUBA3).

Using the same Atlas microarrays, the authors of another study (*Figure 28, Literature, Qian*) compared tissues of 9 palmar nodular superficial fibromatoses with adjacent normal tendon. 16 genes were upregulated, whereas 3 genes showed a reduced expression in diseased tissues. Most of them are involved in the extracellular matrix, including type II alpha collagen (COL2A), decorin (DCN), periostin (POSTN), matrix metalloproteinase 2 (MMP2), amyloid A4 precursor (APP), thymosin, beta 4, X-linked (TMSB4X) and thymosin, beta 10 (TMSB10).

In another study done on cDNA arrays (*Figure 28, Literature, Lee*), tissues derived from 4 palmar cord superficial fibromatoses, four adjacent control fascia and three control palmar fascia were compared with each other. Among the upregulated genes in superficial fibromatoses, the ECM components type V and type VIII collagen, fibronectin (FN), tenascin C (TNC) and serine (or cysteine) proteinase inhibitor, clade H (SERPINH1) were detected. Genes belonging to the biological process of proliferation included maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), TGFβ2 and tenascin.

The gene expression in 6 primary cell cultures derived from palmar superficial fibromatoses was compared with the one in 6 normal palmar fascia fibroblast cell cultures using 2 different microarray platforms (GE Code LinkTM and IlluminaTM). Among the 11 genes detected to be downregulated in diseased cultures on both platforms, the ECM componets type XV collagen, proteoglycan 4 (PRG4) and fibulin-1 (FBLN1) could be found. PRG4 has also been implicated in the regulation of proliferation (*Figure 28, Literature, Satish*).

Another cDNA microarray analysis was performed with the emphasis to detect differentially expressed TGFβ isoforms between palmar cord superficial fibromatosis tissues and adjacent control fascia (*Figure 28, Literature, Zhang*). A dominant increase of TGFβ2 (TGFB2) expression was seen in the diseased tissues, whereas TGFβ1 (TGFB1) and TGFβ3 (TGFB3) were not found to be differentially expressed. In addition, 20 novel differentially expressed genes could be detected. Most of them belong to the ECM, including fibroblast activation protein (FAP), secreted protein, acidic, cysteine-rich (SPARC), thrombospondin 2 (THBS2), type V and VIII collagen, or to the biological process proliferation (ras oncogene family member 31 (RAB31), protein regulator of cytokinesis (PRC1), FAP, MAFB, SPARC).

5.3. Single genes differentiating the two tumors from each other

16 genes that outstandingly differentiate the two tumors from each other by a high ratio and a highly significant p-value were presented in *Figure 17* (S*ection 4.4.3.*). In this section, their cellular functions, if known in context with tumorigenesis, should be discussed.

5.3.1. Genes overexpressed in superficial fibromatosis

ANGPTL7 is a promoter of angiogenesis by enhancing the proliferation of endothelial cells. Its expression can be induced by TGFβ. In turn, it increases the expression of type I collagen. ANGPTL7 has been brought into context with the pathogenesis of glaucoma (Kuchtey *et al.*, 2008). TNMD is related to chondromodulin, which is a cartilage-specific protein that functions to stimulate chondrocyte growth and to inhibit

tube formation of endothelial cells, thus possesses angiogenesis inhibiting properties. A role for this protein in tumorigenesis has not been described to date. DCD is a secreted protein that acts as a proliferation and survival factor in a variety of cancer cell lines under hypoxia or oxidative stress (Stewart *et al.*, 2008). PRG4 is a proteoglycan synthesized by chondrocytes located at the surface of articular cartilage. It functions as a boundary lubricant. Its expression is found in various sarcomas including low grade fibromyxoid sarcoma, malignant fibrous histiocytoma, synovial sarcoma, Ewing tumors and extraskeletal myxoid chondrosarcomas (Panagopoulos *et al.*, 2004). DMRT2 is a transcription factor playing essentials roles during embryonic somite patterning. Recently, it has been brought into context with a translocation occurring in B-cell chronic lymphocytic leukemia (Russel *et al.*, 2009; Seo *et al.*, 2006). As ANGPTL7, MYOC has been associated with the formation of glaucoma (Kim *et al.*, 2001). Normally, it is expressed in many occular tissues and exerts cytoskeletal functions. The secreted protein NOV associates with the extracellular matrix and plays an important role in cardiovascular and skeletal development, as well as fibrosis. In context of tumorigenesis, this protein has been investigated as a potential tumor suppressor in Ewing sarcoma (Perbal *et al.*, 2009), chronic myeloid leukemia (McCallum *et al.*, 2009), and melanoma (Fukunaga-Kalabis *et al.*, 2008). There's a review article about its function in cancer (Perbal, 2006). THBS4 is a secreted glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. It positively influences the TGFβ signalling pathway by associating with ECM-immobilized TGFβ and disrupting the connection between the ECM and TGFβ (Young and Murphy-Ullrich, 2004). Its function in tumorigenesis has not been analyzed, yet. In a recent report, thrombospondins, including THBS4, were shown to activate EGFR in a MMP9-dependent manner, leading to increased epidermal cell migration (Liu *et al.*, 2009).

5.3.2. Genes upregulated in aggressive fibromatosis

The protein encoded by the FGA gene is the alpha component of the blood-borne glycoprotein fibrinogen. Following injury, fibrinogen is cleaved by thrombin to form fibrin which is the most abundant component of blood clots. Mutations in this gene are associated with several disorders including dysfibrinogenemia, hypofibrinogenemia and renal amyloidosis. The gene ACTN2 encodes a muscle-specific form of the cytoskeletal protein actinin. In non-muscle cells, actinins are involved in connecting actin to the cell membrane, whereas in muscle cells, they help to anchor the myofibrillar actin filaments to the Z-disc. A link to tumorigenesis hasn't been published, yet. AREG is a member of the epidermal growth factor (EGF) family that binds to and activates EGFR and is therefore also an activator of the PI3K-AKT pathway. Its contribution to tumorigenesis has been discussed in breast cancer (Sternlicht and Sunnarborg, 2008). APCDD1 was found to be upregulated in colon cancer cell lines and downregulated by APC. Therefore, it was proposed to be involved in the pathogenesis of this disease (Takahashi *et al.*, 2002). The protein encoded by the gene TTN, Titin, is highly expressed in striated muscle cells where it is involved as an adhesion template in the assembly of the contractile machinery. There is no literature available concerning its association with cancer. The transcription factor PITX2 is downstream of the NODAL-signalling route within the TGFβ signalling pathway and has important functions during embryogenesis (left-right axis determination, mesoderm and endoderm induction). Its promoter hypermethlyation has proven to be a marker of poor prognosis in prostate cancer (Weiss *et al.*, 2009) and breast cancer (Nimmrich *et al.*, 2008), thus indicating its

tumor suppressive capabilities. GREM1 is a secreted protein that binds to BMPs and inhibits the association with their receptors, therefore functions as an inhibitor of the BMP-induced TGFβ signalling pathway. In addition, it is a target gene of the canonical Wnt signalling pathway. It is believed that GREM1 is an important constituent of tumor stroma, by providing a favourable microenvironment for cancer cell survival and proliferation in many cancers (Sneddon *et al.*, 2006). WNT5A is an activating ligand for both the canonical and non-canonical Wnt signalling pathways. It has been associated with the pathogenesis of many cancers, by acting both as a tumor promoter and tumor repressor, depending on the type of cancer. The introduction to *Chapter III* is dedicated to the discussion of this protein.

5.4. Detailed analysis of signalling pathways and comparison with published data

In this study, the functional annotation of the selected 2'429 genes differentiating aggressive and superficial fibromatoses from reference fibrous tissue and distinguishing the two tumors from each other revealed that components of the canonical Wnt, the TGFβ, and the PI3K-AKT signalling pathway are statistically overrepresented. In the subsequent detailed analyses of those components, they were grouped according to their functions within the pathways into pathway activity inducers, inhibitors and pathway target genes. This approach aimed to deduce informations about differential activities within these pathways between the tissues analyzed. Applying such an approach, the following restrictions of the validity of the resulting informations must be considered.

1) The numerical analysis of differentially expressed pathway inducers and inhibitors does not necessarily lead to a conclusive statement about the differential activity within the pathway, because the single components may exert variable impacts.
2) The activity of single components is often regulated on a post-translational level, e.g. through phosphorylations.
3) The expression level of a certain target gene is mostly the result of a crosstalk between several different signalling pathways and therefore gives a hint at the differential activity of this pathway, but does not proof it.
4) Even though carefully selected by light microscopy, surgically removed tumor tissue also contains other tissue components, e.g. the tumor stroma, that might contribute to the expression profile.
5) Genome wide expression profiling yields only a momentary picture of a continuously changing spectrum of transcriptional activiy.

5.4.1. The canonical Wnt signalling pathway

A hallmark in the diagnosis of fibromatoses represents the nuclear accumulation of β-catenin in the nucleus, indicating an increased TCF-dependent transcription in these tumors (*Section 2.2.1.*). In this study, the functional annotation of the selected genes differentiating aggressive and superficial fibromatoses from reference fibrous tissue and distinguishing the two tumors from each other revealed a statistically significant overrepresentation of components of the canonical Wnt signalling pathway. *Gene lists A* and *B* in the *Appendix* allow to compare those results with findings published in the literature. The subsequent analysis of the identities of the differentially

expressed components point towards a higher activity of this pathway in fibromatoses as compared to reference fibrous tissues, the existence of a negative feedback loop mechanism established in fibromatoses to try to counterbalance an excessive pathway activity, and reveals a high overlap of deregulated pathway components between aggressive and superficial fibromatoses.

- Higher activity in fibromatoses as compared to reference fibrous tissues

In aggressive fibromatosis, more pathway activity inducers are overexpressed (10) than in the reference fibrous tissue (6). In addition, the number of differentially expressed canonical Wnt signalling target genes reflecting an increased activity of this pathway in aggressive (21) and superficial fibromatoses (14) is clearly higher than in the reference fibrous tissue (9 and 7, respectively). Findings that support the current view, that fibromatoses are characterized by an increased activity in this pathway (*Section 2.2.1.*).

Such a comprehensive analysis of differentially expressed canonical Wnt signalling pathway components in fibromatoses in comparison to reference fibrous tissues has not been published, yet.

Among the pathway activity inducers, only a few of them have already been mentioned in the literature to be differentially expressed in fibromatoses. For example, among the several Wnt ligands and Frizzled receptors found to be differentially expressed in this study, only WNT5A has ever been found to be overexpressed in aggressive fibromatosis. In a microarray study comparing the gene expression in primary cell cultures of aggressive fibromatoses with the one in reference fibroblast cultures (Denys *et al.*, 2004b).

In several independent studies, the overexpressions of the canonical Wnt signalling target genes MMP3 (Denys *et al.*, 2004a; Denys *et al.*, 2004b; Kong *et al.*, 2004), MMP7 (Denys *et al.*, 2004b), PTGS2 (Poon *et al.*, 2001), CCND1 (Saito *et al.*, 2002; Saito *et al.*, 2001), CCND2 (Denys *et al.*, 2004b), EPHB3 (Skubitz and Skubitz, 2004) and WISP1 (Skubitz and Skubitz, 2004) have been determined in aggressive fibromatosis, whereas in superficial fibromatosis, an increased expression of FN1 (Kosmehl *et al.*, 1995; Tomasek *et al.*, 1986), MMP2 (Qian *et al.*, 2004) and MYC (Jemec *et al.*, 1999) was observed. In our study, an overexpression of MMP3 and MYC (downregulated in both types of fibromatoses) could not be verified, whereas the results for the other target genes could be confirmed. A downregulation of MYC may reflect the dominant effect of an active TGFβ signalling pathway in these tissues, since the TGFβ signalling pathway exerts its negative impact on cell proliferation in part through a downregulation of MYC (*Section 1.2.2.*).

- *A negative feedback loop mechanism established in fibromatoses may try to counterbalance an excessive pathway activity*

The study revealed that there is a bias in the regulation of pathway activity inhibitors towards their higher expressions in fibromatoses. 8 pathway activity inhibitors are upregulated in aggressive fibromatosis, whereas none could be found to be overexpressed in the reference tissue. In superficial fibromatosis, 5 inhibitors are overexpressed, but only 1 in the reference fibrous tissue. This may reflect the existence of a negative feedback loop mechanism established in fibromatoses to try

to counterbalance a canonical Wnt signalling activity that ran out of control. Indeed, the pathway activity inhibitors AXIN2 and SFRP2 are established canonical Wnt signalling target genes, but have not been described to be upregulated in fibromatoses, yet. The same accounts for the other activity inhibitors found to be enhanced in their expressions in fibromatoses (DKK2, DKK3, APC, APC2, SFRP4, SOX17, WIF1).

- High overlap of deregulated pathway components between tumors

Half of the pathway components found to be deregulated in aggressive fibromatosis are in the same way regulated in superficial fibromatosis. And even three fourth of the differentially expressed components detected in superficial fibromatosis are equally regulated in aggressive fibromatosis. This finding suggests common mechanisms being responsible for this outcome. Whereas for aggressive fibromatosis, APC and β-catenin mutations are accountable for a deregulation of the canonical Wnt signalling pathway in most of the cases, the causing mechanisms in superficial fibromatoses remain to be elucidated (S*ection 2.2.1.*).

5.4.2. The TGFβ signalling pathway

The results of several studies suggest a contribution of a deregulated TGFβ signalling pathway activity to the pathogenesis of superficial fibromatosis (S*ection 2.2.2.*). The functional annotation of the selected genes in this study revealed that a statistically significant high number of TGFβ signalling pathway components are differentially expressed between fibromatoses and reference fibrous tissues, as well as between the two tumors. *Gene lists A* and *B* in the *Appendix* allow to compare those results with findings published in the literature. The examination of the identities of the deregulated components supports the notion that the TGFβ signalling pathway shows a higher activity in both types of fibromatoses as compared to reference fibrous tissues and demonstrates that there is a high overlap of deregulated pathway components between the two tumors.

- Higher activity in fibromatoses as compared to reference fibrous tissues

In both aggressive (10) and superficial fibromatosis (8), more TGFβ pathway activity inducers are overexpressed as compared to the reference fibrous tissue (6 and 4, respectively).

The impression of an enhanced activity of the TGFβ signalling pathway in fibromatoses is further underlined by the analysis of differentially expressed pathway target genes. The number of target genes reflecting an increased activity of this pathway in aggressive (10) and superficial fibromatosis (9) is higher than in the reference fibrous tissue (2 and 5, respectively).

Concerning the upstream pathway inducers, our data confirm findings published in the literature whereupon TGFB3 is more highly expressed in both types of fibromatoses as compared to reference fibrous tissues (Berndt *et al.*, 1995; Skubitz and Skubitz, 2004; Zhang *et al.*, 2008). In addition, an overexpression of TGFB2 (Badalamente *et al.*, 1996; Berndt *et al.*, 1995; Kuhn *et al.*, 2002; Lee *et al.*, 2006; Zhang *et al.*, 2008) and MMP2 (Qian *et al.*, 2004) in superficial fibromatosis could be verified. Contradictory to data in the literature (Locci *et al.*, 2001), our data reveal a

downregulation of TGFBR2 in aggressive fibromatosis and of TGFBR3 in both fibromatoses. In addition, the determined overexpression of BMP7 in both fibromatoses and of MMP2 in aggressive fibromatosis is not in agreement with data published in the literature (Denys *et al.*, 2004a; Kong *et al.*, 2004; Shin *et al.*, 2004). All other pathway inducers found to be differentially expressed in our study have not been mentioned in the literature in context with fibromatoses so far, namely anti-Mullerian hormone (AMH), BMP8A, thrombospondins 2 (THBS2) and 4 (THBS4), galectin 3 (LGALS3), inhibin β A and B (INHBA, INHBB), nodal, activin A receptor type IB (ACVR1B), mothers against decapentaplegic homolog 3 (SMAD3) and early growth response 1 (EGR1).

Concerning the ECM component target genes of this pathway, an overexpression in both fibromatoses has been reported for COL1A1 (Kopp *et al.*, 2006; Skubitz and Skubitz, 2004) and COL3A1 (Howard *et al.*, 2003; Skubitz and Skubitz, 2004), whereas an enhanced expression of COL1A2 (Kopp *et al.*, 2006), fibronectin (FN1) (Berndt *et al.*, 1995; Tomasek *et al.*, 1986) and TGFβ-induced (TGFBI) (Kraljevic Pavelic *et al.*, 2009) has been specifically associated with superficial fibromatoses. The overexpressions of osteoglycin (OGN), osteonectin (SPARC) and collagen triple helix repeat containing 1 (CTHRC1) have not been described in the literature, yet. The single ECM target gene found to be upregulated in the reference tissue is TGFβ inducible nuclear protein 1 (TINP1) that has not been brought into context with fibromatoses, yet.

The TGFβ signalling pathway achieves its negative effect on proliferation by inhibiting the expression of c-myc (MYC) and inducing the expression of the cyclin / cyclin-dependent kinase complex inhibitor 1B (CDKN1B). In both fibromatoses, those two proteins were found to be downregulated. The observed downregulation of CDKN1B may reflect a crosstalk with another pathway that exerts a dominant inhibiting effect. There exist no data concerning the expression of CDKN1B in fibromatoses. MYC has been described to be upregulated in superficial fibromatosis as compared to normal fascia (Jemec *et al.*, 1999), a finding that is therefore contradictory to our data.

Another target gene found to be upregulated specifically in aggressive fibromatosis represents the transcription factor PITX2. Its promoter hypermethylation has been associated with poor prognosis in prostate cancer (Weiss *et al.*, 2009) and breast cancer (Nimmrich *et al.*, 2008), thus indicating its tumor suppressive capabilities in these tumors. Up to now, is has not been described in relation to the pathogenesis of fibromatosis.

The TGFβ signalling pathway induces apoptosis in part through the upregulation of growth arrest and DNA damage inducible (GADD45), whose expression is found to be downregulated in superficial fibromatosis, therefore indicating another possible crosstalk with unknown pathways. In the literature, it has not been discussed in context with fibromatoses so far.

Two other target genes of the TGFβ signalling pathway that are specifically downregulated in superficial fibromatosis are inhibitor of DNA binding 2 (ID2) and 4 (ID4). Other members of this family of transcription factors, ID1 and ID3, were shown to be essential for the successful formation of breast cancer metastases after the cells entered the lung parenchyma (Gupta *et al.*, 2007). Therefore, the

downregulation of ID2 and ID4, by whatever pathway, may contribute to the inability of superficial fibromatosis to form metastases.

- *The differential expressions of pathway activity inhibitors do not indicate the establishment of a negative feedback loop in fibromatoses*

In both tumors and the reference fibrous tissue, similar numbers of pathway inhibitors are overexpressed (Aggr vs. Ref = 4:3 ; Super vs. Ref = 2:2). This suggests that there exists no negative feedback loop in fibromatoses trying to counteract an exaggerated TGFβ signalling pathway activity.

Of the those differentially expressed pathway inhibitors, only asporin (ASPN) (Skubitz and Skubitz, 2004), cartilage oligomeric matrix protein (COMP) (Skubitz and Skubitz, 2004) and decorin (DCN) (Qian *et al.*, 2004) have ever been mentioned in publications to be regulated in their expressions in fibromatoses. Whereas the statements for ASPN and DCN could be confirmed, the overexpression of COMP in aggressive fibromatosis was not verified by our data. The remaining genes found to be differentially expressed in our study are so far unknown for being regulated in fibromatoses: gremlin 1 homolog (GREM1), cartilage intermediate layer protein (CILP), follistatin (FST), follistatin-like 3 (FSTL3) and BMP and activin membrane-bound inhibitor homolog (BAMBI).

- High overlap of deregulated pathway components between tumors

Similarly to the picture obtained analyzing the canonical Wnt pathway, the examination of the differentially expressed TGFβ pathway genes revealed a pronounced overlap between the deregulated components in the two tumors. Two thirds of the pathway components found to be deregulated in aggressive fibromatosis are in the same way regulated in superficial fibromatosis. And even three fourth of the differentially expressed components detected in superficial fibromatosis are equally regulated in aggressive fibromatosis. The reason for this outcome is unknown, but it suggests a common deregulation inducing mechanism.

5.4.3. The PI3K-AKT signalling pathway

The PI3K-AKT signalling pathway plays a central role in the pathogenesis of diverse cancers by representing an effector pathway for several important growth factors such as EGF, IGF, HGF or PDGF to regulate proliferation, apoptosis, angiogenesis, protein synthesis and glycogen synthesis (*Section 1.3.*).

The DAVID-based functional annotation of the differentially expressed genes in our study associated this pathway with a possible role in the pathogenesis of fibromatoses, since it is overrepresented in the list of genes commonly differentiating the two tumors from the reference fibrous tissue. *Gene lists A* and *B* in the *Appendix* allow to compare those results with findings published in the literature.

The central component of this pathway, AKT, is not a transcription factor, but a serine/threonine-kinase modulating the activities of a plethora of downstream targets on the protein level by phosphorylation (*Section 1.3.1.*, *Figure 5*). These proteins in turn transmit the signal on the protein level to other target proteins or regulate the transcription of target genes (in the case of transcription factors), resulting in an

increasing branching and expansion of the downstream signalling pathways. The PI3K-AKT pathway does not contain one major, central transcription factor that alters the transcription of a limited number of target genes, but induces crosstalks to several individual signal transduction routes. Therefore, informations about differential activities in the PI3K-AKT pathway between different tissues can be deduced by enumerating differentially expressed inducers and inhibitors only upstream of AKT. Downstream of AKT, the focus was laid on the analysis of the biological functions of the differentially expressed pathway components in terms of a possible contribution of these genes to the pathogenesis of fibromatoses. The reason for the up- or downregulation of these genes is unknown.

- The analysis of components upstream of AKT reveals no differences in the activity of the PI3K-AKT pathway between fibromatoses and reference fibous tissue

The differential expressions of pathway inducers and inhibitors upstream of AKT do not point towards a differential activity of this pathway between fibromatoses and reference fibrous tissue. Similar numbers of inducers and inhibitors are overexpressed in tumors and in reference tissue.

Of all deregulated components upstream of AKT, only the downregulated insulin-like growth factor-binding protein 6 (IGFBP6) has ever been brought into context with the pathogenesis of fibromatoses (Denys *et al.*, 2004b). Comparing the gene expression of four primary cell cultures of aggressive fibromatoses (no proof of tumoral origin was provided) with the one in cultures of normal fascia using Affymetrix microarrays, the authors of the mentioned study found 69 genes to be differentially expressed. IGFBP6 was highly downregulated in all aggressive fibromatosis cell cultures analyzed, a finding that was confirmed using RT-PCR, Northern and Western blot. IGFBP6 preferentially binds and inhibits insulin-like growth 2 (IGF2) from binding to the insulin-like growth factor 1 receptor (IGF1R). Cell culture proliferation assays revealed that exogenous IGF2 acts as a mitogen in aggressive fibromatoses. This effect could be abrogated by the concomitant treatment with recombinant IGFBP6 or an antibody against IGF1R, indicating that the missing expression of IGFBP6 in aggressive fibromatosis may contribute to their pathogenesis through a deregulated IGF2-IGF1R-dependent proliferation activity. Whether the PI3K-AKT pathway is activated through this proposed mechanism was not analyzed (Denys *et al.*, 2004b).

Even though our approach did not reveal any differential activity in the PI3K-AKT pathway between the tissues analyzed, a direct proof of a deregulated PI3K-AKT pathway activity has recently been delivered in a study done in superficial fibromatosis. An integrated proteomics approach (combining two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) with a bioinformatic tool to depict protein-protein interactions (interactome)) was applied to detect new molecular mechanisms involved in the pathogenesis of superficial fibromatosis. Starting from a list of 28 proteins (involved in biological processes such as extra- and intracellular signalling, metabolism, cytoskeleton and oxidative stress) determined to be overexpressed in tissues from superficial fibromatoses as compared to reference tissues using 2-DE/MS, the in silico interactome map suggested a functional link of v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2 (ERBB2) and insulin-like growth factor receptor 1 (IGFR1) to the 28 in vitro detected differentially expressed proteins. Western blot analysis and immunohistochemistry confirmed their overexpression in superficial fibromatosis. In addition, the interactome approach

indicated an involvement of the PI3K-AKT signalling pathway in the pathogensis of superficial fibromatosis. Western blot analyses approved an overexpression of AKT and its activated form, phospho-AKT, in diseased tissues, pointing towards a higher activity of the PI3K-AKT pathway in superficial fibromatosis as compared to reference tissues (Kraljevic Pavelic *et al.*, 2009). Therefore, at least in superficial fibromatosis, the PI3K-AKT pathway is activated. Of those 28 differentially expressed proteins, four could be verified on the expression level in our study, namely type VI collagen (COL6A3), galectin 1 (LGALS1), TGFβ-induced (TGFBI) and triosephosphate isomerise (TPI1).

- *Differentially expressed genes downstream of AKT inhibit apoptosis in both fibromatoses*

The PI3K-AKT pathway inhibits apoptosis through the AKT-dependent modulation of the function of diverse downstream proteins, both on the protein (phosphorylation, ubiquitination) and the RNA level (transcription) (*Section 1.3.1.*, *Figure 5*).

AKT represses apoptosis by phosphorylating and activating mouse double minute 2 homolog (MDM2) that binds to and inhibits the key apoptosis-inducing transcription factor p53 (TP53). Additionally, AKT induces the activity of the anti-apoptotic signalling pathway nuclear factor of kappa light chain gene enhancer in B-cells (NFκB), by phosphorylating and activating the inhibitor of NFκB kinase (IKK) that in turn phosphorylates and inhibits the inhibitor of NFκB (NFKBI). Finally, it phosphorylates and inhibits forkhead box (FOXO) family transcription factors, known for their transcriptional induction of apoptosis-inducing genes.

Key components on the above described AKT-dependent apoptosis repressing routes are accordingly regulated on the transcriptional level in both fibromatoses as compared to the reference fibrous tissue. NFKBIA, FOXO1A and TP53, all potent inducers of apoptosis, are downregulated in both fibromatoses. Their transcriptional inhibition, induced by whatever mechanism, may contribute to the pathogenesis of fibromatoses. Perhaps, the function of those three proteins is further repressed on the protein level by an increased activity of the PI3K-AKT pathway in fibromatoses, as it is described in the above mentioned paper for superficial fibromatosis (Kraljevic Pavelic *et al.*, 2009).

A differential expression of NFKB1A and FOXO1A in fibromatoses in comparison to reference tissues has not been mentioned in the literature, yet. In one report, the authors describe the analysis of the expression of TP53 both on the protein (IHC) and the mRNA level (RT-PCR) in aggressive and superficial fibromatoses. They could not find a differential expression, confirming our results (Muller *et al.*, 1996)..

- *Differentially expressed genes downstream of AKT induce proliferation in both fibromatoses*

The PI3K-AKT pathway induces induces proliferation through the AKT-dependent modulation of the function of diverse downstream proteins, both on the protein (phosphorylation, ubiquitination) and the RNA level (transcription) (*Section 1.3.1.*, *Figure 5*).

AKT stimulates cell proliferation via phosphorylation and inhibition of GSK3β and members of the FOXO family of transcription factors. By inactivating GSK3β, AKT reduces GSK3β-mediated phosphorylation and inactivation of cyclin D1 (CCND1) and c-myc (MYC), two central components for cell cycle progression. In addition, inhibition of GSK3β leads to an accumulation of β-catenin and an activation of TCF-dependent transcription, whereupon the transcription of CCND1 and MYC is increased. Through the phosphorylation and inhibition of FOXO transcription factors, the PI3K-AKT pathway represses the expression of the cell cycle progression inhibitor cyclin-dependent kinase inhibitor CDKN1B (p27, inhibits cyclin E/A – CDK2 complexes), thus further accelerates proliferation.

Three genes involved in the above described regulation of proliferation are differentially expressed in both fibromatoses, namely CDKN1B, CCND1 and MYC. CDKN1B was found to be downregulated, whereas CCND1 was upregulated, reflecting the effect of an active PI3K-AKT pathway on their transcription. In addition, an active PI3K-AKT-pathway in fibromatoses may increase the function of CCND1 on the protein level, by releaving it from the GSK3β-mediated phosphorylation and inhibition. In contrast, the inducer of proliferation MYC was found to be downregulated in its expression in both tumors as compared to the reference fibrous tissue. This may reflect the result of an inhibiting effect of the TGFβ signalling pathway on the expression of MYC (*Section 1.2.2.*). Another inducer of proliferation, β-catenin, was shown to be specifically upregulated on the transcriptional level in aggressive fibromatosis. Its observed overexpression, together perhaps with an active PI3K-AKT signalling-dependent repression of its degradation, may contribute to an increased proliferation of aggressive fibromatosis tumor cells. Overall, the differential expressions of those PI3K-AKT pathway components involved in regulating proliferation, induced by unknown mechanisms, indicate a proliferation enhancing effect in fibromatoses. Therefore, they may play a role in the pathogenesis of fibromatoses.

β-catenin is known for its nuclear accumulation in both fibromatoses (Alman *et al.*, 1997a; Carlson and Fletcher, 2007; Ferenc *et al.*, 2009; Lazar *et al.*, 2008; Montgomery *et al.*, 2001; Saito *et al.*, 2001; Tejpar *et al.*, 1999; Varallo *et al.*, 2003), On the mRNA level, an overexpression has been described in aggressive fibromatoses carrying β-catenin mutations as compared to those without mutations (Saito *et al.*, 2002). CCND1 is known to be overexpressed in aggressive fibromatoses (Saito *et al.*, 2002; Saito *et al.*, 2001). MYC was shown to be upregulated in superficial fibromatoses (Jemec *et al.*, 1999), a finding that is in disagreement with our results. The differential expression of CDKN1B has not been mentioned in the literature in context of fibromatoses, yet.

- *The major inducer of angiogenesis, HIF1α, is upregulated in aggressive fibromatosis*

The PI3K-AKT pathway induces angiogenesis by upregulating the transcription of hypoxia-inducible factor 1 α subunit (HIF1α) (*Section 1.3.1.*, *Figure 5*).

In our study, HIF1α was found to be more highly expressed in aggressive fibromatosis as compared to reference fibrous tissue, indicating its role in the

pathogenesis of this tumor. In context of fibromatosis, this protein has not been mentioned in the literature up to date.

In contrast, a target gene of HIF1α, vascular endothelial growth factor (VEGF), is downregulated in superficial fibromatosis as compared to the reference fibrous tissue. Its relevance for the pathogenesis of fibromatoses has not been discussed in the literature, yet.

- High overlap of deregulated pathway components between tumors

The PI3K-AKT pathway is characterized by a high number of differentially expressed components between fibromatoses and normal fibrous tissues, and concurrently by a pronounced overlap of the deregulated pathway components between the two fibromatoses. Two thirds of the pathway components found to be deregulated in aggressive fibromatosis are in the same way regulated in superficial fibromatosis. And even nearly three fourth of the differentially expressed components detected in superficial fibromatosis are equally regulated in aggressive fibromatosis. The high similarity in the way of deregulation of this pathway in the two tumors suggests that common, so far unknown mechanisms are responsible for this outcome.

5.5. The process of wound healing

In *Section 1.4.*, a brief introduction in the process of wound healing was given. During the proliferative phase of wound healing, fibroblasts are involved in the formation of the granulation tissue, by increasing their proliferation rate, transforming into myofibroblasts and invading into the wound space. There, they deposit an abundant collagen matrix needed to support further cell ingrowth and to stabilize and contract the wound. Increased TCF-dependent transcription, transactivated by TGFβ- and EGF-induced signalling pathways, was shown to be involved in this process. These parallels concerning histology, cellular basis and involved signalling pathways between fibromatoses and the process of wound healing led to the theory that the pathogenesis of aggressive (Cheon *et al.*, 2005; Cheon *et al.*, 2002; Tejpar *et al.*, 2005) and superficial fibromatosis (Cordova *et al.*, 2005; Kloen, 1999) is based on mechanisms being responsible for normal wound healing. Whereas in normal wound healing, a negative feed-back loop controls an exaggerated process, molecular alterations in tumor cells of fibromatoses may account for its continuous action.

Our data point towards a higher activity of both the canonical Wnt and the TGFβ signalling pathway in fibromatoses as compared to reference fibrous tissues. In addition, they suggest the establishment of a negative feedback loop in fibromatoses to try to counterbalance an exaggerated canonical Wnt signalling pathway activity. Altogether, our data therefore underline the proposed parallels between fibromatoses and the process of wound healing.

5.6. Differentially expressed genes belonging to the other biological processes

The differentially expressed genes belonging to the functional process extracellular matrix (ECM) can be be found in the *Appendix*, *Gene lists A* and *B*. There, a comparison with literature data can also be obtained. Numerous reports using different experimental techniques such as gene expression array, immunohistochemistry, in situ hybridization or Western blot describe an overexpression of ECM components in fibromatoses. Collagen type I (Kopp *et al.*, 2006; Naito *et al.*, 1998; Skubitz and Skubitz, 2004; Zamora *et al.*, 1994), type III (Howard *et al.*, 2003; Naito *et al.*, 1998; Skubitz and Skubitz, 2004), type V (Lee *et al.*, 2006; Skubitz and Skubitz, 2004) and type VI (Denys *et al.*, 2004b; Kraljevic Pavelic *et al.*, 2009; Magro *et al.*, 1995a; Thurston, 2003; Zhang *et al.*, 2008) were shown to be overexpressed in both types of fibromatoses as compared to reference fibrous tissues. Collagen type XI, XII, XIV, spondin 2, biglycan, cartilage oligomeric protein (COMP) and versican were found to be upregulated in aggressive fibromatosis (Skubitz and Skubitz, 2004), whereas superficial fibromatoses were characterized by an overexpression of type II (Qian *et al.*, 2004), type IV (Berndt *et al.*, 1994; Kosmehl *et al.*, 1995; Magro *et al.*, 1997), and type VIII collagen (Lee *et al.*, 2006), fibronectin (Berndt *et al.*, 1995; Halliday *et al.*, 1994; Howard *et al.*, 2004; Kosmehl *et al.*, 1995; Lee *et al.*, 2006; Tomasek *et al.*, 1986), laminin β1, β2 (Kosmehl *et al.*, 1995) and tenascin C (Lee *et al.*, 2006; Shih *et al.*, 2009).

The differentially expressed genes belonging to the remaining functional processes, including ECM-receptor interaction, adherens junction and cell adhesion molecules (CAMs), proliferation, cytoskeleton and complement and coagulation cascades can be found in the *Appendix*, *Gene lists A* and *B* as well. In addition, the genes categorized as ‘others’, including those that could not be associated with a biological process. Genes that have been mentioned in the literature as being differentially expressed between the tissues analysed, are marked with the corresponding informations in the first column.

Chapter II
Establishment of primary cell cultures derived from fibromatoses

1. General introduction

Cells directly cultured from a tissue biopsy are known as primary cultures. Two barriers prevent cultured cells from proliferating indefinitely in culture: senescence and crisis.

Senescence is characterized by the cessation of proliferation after a certain number of replicative doublings (~50-60), depending on the species from which the cells were derived, on the tissue of origin, and on the age of the donor organism. Cells in the stadium of senescence normally retain viability for a prolonged time. Senescence is provoked by physiologic stresses that cells are faced to in vitro, such as fluctuations of oxygen tension leading to cumulative oxidative damage. In the great majority of human tumors, the tumor suppressor genes tumor protein p53 (TP53) and retinoblastoma 1 (RB1) are inactivated through mutations. This allows tumor cells in vivo to adapt to a variety of physiologic stresses normally leading to cessation of proliferation and even apoptosis. Likewise, these mutations help tumor cells taken into culture to resist to many of the stresses imposed by in vitro culture conditions.
Senescence of cultured human fibroblasts and epithelial cells can be avoided if the large T oncoprotein of the simian virus SV40 is expressed in these cells. By sequestering both the TP53 and RB1 tumor suppressor proteins, the large T oncoprotein is able to neutralize the key cellular signalling pathways leading to senescence in response to stress.

While cultured cells being mutated at TP53 and RB1 or expressing the large T oncoprotein succeed in bypassing senescence, they still will not be immortalized. After an additional number of replicative doublings (~10-20) beyond the number at which they would usually senesce, the cells enter into crisis and exhibit widespread apoptosis. Crisis is provoked by the erosion of telomeres, which results in end-to-end chromosomal fusions and karyotypic chaos. The telomeres are located at the ends of the chromosomes and act to prevent the end-to-end fusion of chromosomal DNA molecules. While the replication machinery that operates during the S phase of the cell cycle is highly effective at copying the sequences in the middle of linear DNA molecules, this machinery has great difficulty in copying sequences at the very ends of these molecules. This end-replication problem provides a molecular explanation for the observed shortening of telomeric DNA each time a normal human cell passes through a cell cycle. Telomere regeneration can be accomplished through the action of the telomerase enzyme, which functions specifically to elongate telomeric DNA. Telomerase activity is clearly detectable in 85-90% of human tumor cell samples, while being present at very low levels in the lysates of most types of normal human cells. The telomerase enzyme is a complex composed of a number of distinct subunits. The core contains two subunits: a reverse transcriptase and a RNA template. The reverse transcriptase is encoded by the gene human telomerase reverse transcriptase (hTERT) that needs a short segment of the RNA template, encoded by the telomerase-associated RNA molecule gene (hTR), to accomplish

telomere regeneration. Before entering crisis, human cells are essentially telomerase activity negative and do not express appreciable levels of the hTERT mRNA. Exogenous expression of hTERT in cells just before they are destined to enter crisis confers telomerase activity to these cells, causes elongation of their greatly shorted telomeres, prevents entrance into crisis, and allows such cells to grow indefinitely. In fact, cell immortalization is thought to be a step that governs the development of all human cancers. The mechanisms that lead to the de-repression of hTERT transcription during tumor progression are complex and still not fully understood. Multiple transcription factors appear to collaborate to activate the hTERT promoter. c-myc (MYC) that exerts a central role in the activation of the cell cycle, also contributes to the transcription of hTERT. Therefore, activating mutations within the MYC gene that can be often found in human tumors, may contribute to a constitutive expression of hTERT leading to immortalization.

Cells that can be passaged indefinitely in culture and therefore are able to prevent both senescence and crisis, constitute a cell line. Nevertheless, cancer cells often fail to adapt to culture, because in vivo they strongly depend on several other cell types, the so called tumor stroma, supporting their viability and proliferation (Weinberg, The Biology of Cancer, 2007, Garland Science, New York).

2. Introduction

An essential step after the establishment of a primary tumor cell culture is to formally prove the derivation from the original tumor in vivo. This can often be done by the analysis of a certain mutation present in the tumor cells of the original tumor tissue which should be present in the primary tumor cell culture as well. Alternatively, certain marker proteins may exist that distinguish the tumor cells from tumor-associated cells.

2.1. Markers applicable to aggressive and superficial fibromatoses

Aggressive fibromatosis is a monoclonal proliferartion of myofibroblast-like tumor cells (Alman *et al.*, 1997b; Li *et al.*, 1996). Aggressive fibromatoses are in most of the cases characterized by a monoallelic mutation in the β-catenin or a biallelic mutation in the APC-gene (Lazar *et al.*, 2008; Tejpar *et al.*, 1999) (*Chapter I, Section 2.2.1.*). The derivation of primary cell cultures from tumor cells can therefore be proven by the detection of identical mutations as present also in the tumor tissue.

In superficial fibromatoses, causing mutations have not been found, yet (Montgomery *et al.*, 2001; Varallo *et al.*, 2003). Whether they represent a true neoplastic proliferation or rather a polyclonal reactive process is controversially discussed. Two studies report a polyclonal pattern of X chromosome inactivation of the androgen receptor gene in superficial fibromatosis tissues from two and eight women, respectively, thus favouring a polyclonal reactive process (Chansky *et al.*, 1999; Wang and Zhu, 2006). Others describe clonal chromosomal aberrations at variable frequencies in patients suffering from this disease, supporting the notion of a neoplastic process (Dal Cin *et al.*, 1999; De Wever *et al.*, 2000).

In vivo, the tumor cells of fibromatoses exhibit features of myofibroblasts as indicated by the expression of bundles of smooth muscle actin (α-SMA) microfilaments (similar to those found in smooth muscle cells), differentiating them from tumor-associated normal fibroblasts. But in vitro, α-SMA is not useful as tumor cell marker, because normal fibroblasts are characterized by α-SMA positive stress fibres as well.

Since no reliable genetic or phenotypic markers exist in superficial fibromatoses, the derivation of primary cell cultures from the original tumor tissue can not be proven to date.

2.2. Published studies using primary cultures of aggressive and superficial fibromatoses

Several studies published in the literature report the establishment of primary tumor cell cultures derived from fibromatoses for the use in functional studies.

2.2.1. Aggressive fibromatosis

In the following three reports, the authors claimed that primary cell cultures derived from aggressive fibromatoses are characterized by an increased activity of the Wnt signalling pathway. In the first study, the experiments were based on cell cultures derived from tumors carrying somatic APC mutations. Transient transfection of these cells with an APC expression plasmid led to a decrease of the β-catenin protein level (Western blot) and a diminished proliferation rate. A concomitant expression of a constitutive active form of β-catenin (ΔN89-β-catenin, missing the N-terminal phosphorylation sites) abrogated the negative effect of APC on the cellular proliferation, indicating that APC controls the proliferation of these cells through its contribution to the degradation process of β-catenin (Li *et al.*, 1998). In a subsequent study, primary cell cultures of aggressive fibromatoses were reported to be characterized by a pronounced nuclear accumulation of β-catenin in comparison to normal control fibroblasts (Tejpar *et al.*, 1999). In a further study, the authors claim that primary aggressive fibromatosis cell cultures are characterized by an increased TCF-dependent transcriptional activity as compared to cell cultures of normal fibroblasts. In addition, they could show that in aggressive fibromatosis, β-catenin transactivates TCF-dependent transcription by binding to TCF3, not TCF4 as in colon carcinoma (Tejpar *et al.*, 2001).

Using primary cell cultures of FAP-associated aggressive fibromatoses and cell cultures of normal fibroblasts, the authors of another study found an overexpression of TGFβ1 (ELISA, Northern blot) and its type I and II receptors (TGFBR1, TGFBR2), (SDS-PAGE and fluorography) in the tumor cells. Tumor cell derived TGFβ1 was shown to induce the secretion of TNFα in monocytes, that in turn stimulates the tumor cells to produce the extracellular matrix (ECM) component glycosaminoglycan (GAG). Treatment of the tumor cells with Tamoxifen reduced their secretion of TGFβ1 and GAG. In addition, it inhibited the secretion of tumor necrosis factor α (TNFα) in monocytes. Therefore, the authors propose that a TGFβ1-induced crosstalk between tumor cells and infiltrating monocytes may contribute to the pathogenesis of aggressive fibromatosis, by stimulating the tumor cells to produce ECM components (Locci *et al.*, 2001).

In another study, insulin-like growth factor-binding protein 6 (IGFBP6) was shown to be highly downregulated in aggressive fibromatosis cell cultures as compared to cell cultures of normal fibroblasts (Microarray, RT-PCR, Northern and Western blot). IGFBP6 preferentially binds and inhibits insulin-like growth 2 (IGF2) from binding to the insulin-like growth factor 1 receptor (IGF1R). Cell culture proliferation assays revealed that exogenous IGF2 acts as a mitogen in aggressive fibromatoses. This effect could be abrogated by the concomitant treatment with recombinant IGFBP6 or an antibody against IGF1R, indicating that the missing expression of IGFBP6 in aggressive fibromatosis may contribute to their pathogenesis through a deregulated IGF2-IGF1R-dependent proliferation activity (Denys *et al.*, 2004b).

Using a type I collagen cell invasion assay, it was reported that cell cultures derived from aggressive fibromatoses show an invasive capacity that could be further stimulated by the addition of conditioned medium. The invasion of tumor cells into the collagen gel could be partly inhibited by a synthetic matrix metalloproteinase (MMP) inhibitor, suggesting a critical role for MMPs in this locally invasive tumor (Denys *et al.*, 2004a).

The authors of another study report the overexpression of the Wilms' tumor gene 1 (WT1) in primary cell cultures of aggressive fibromatoses as compared to cell cultures of normal fibroblasts (RT-PCR, Western blot, Immunohistochemistry) (Amini Nik *et al.*, 2005).

2.2.2. Superficial fibromatosis

Most experiments with primary cell cultures derived from superficial fibromatoses were aimed to analyze the impact of TGFβ stimulation. A positive effect of TGFβ1 and TGFβ2 on the proliferation of superficial fibromatosis tumor cells was reported, whereas cultures of normal fibroblasts did not react (Badalamente *et al.*, 1996). This result was indirectly proven by an experiment showing that TGFβ2 increases the DNA and protein synthesis in cultures of superficial fibromatoses (Kuhn *et al.*, 2001). TGFβ1 enhanced the proportion of α-SMA-positive cells in cultures of superficial fibromatoses, whereas normal fibroblasts did not respond (IHC) (Bisson *et al.*, 2003). In another cell culture experiment, TGFβ1 was shown to induce the expression of α-SMA, type I collagen and plasminogen activator inhibitor type I (PAI-1) (Western blot) (Kopp *et al.*, 2006). Primary tumor cells of superficial fibromatoses contracted fibroblast-populated collagen lattices significantly more than control fibroblasts (Kuhn *et al.*, 2002). The addition of TGFβ1 (Bisson *et al.*, 2009) and TGFβ2 (Tse *et al.*, 2004) increased the generated force in both control and diseased cells.

In another study, treatment of superficial fibromatosis tumor cells with 5α-dihydrotestosterone increased their proliferation and expression of α-SMA (Pagnotta *et al.*, 2003).

Tumor cells derived from superficial fibromatoses and normal palmar fascia fibroblasts were analyzed for mRNA expression of bone morphogenetic proteins (BMPs) and their receptors (BMPRs) using RT-PCR. The expression levels of BMP4, BMP6, BMP8, BMPR1A, BMPR1B and BMPR2 were found to be reduced in tumor cells as compared to normal fibroblasts. The differential expressions of BMP4 and BMP8 could be confirmed on the protein level (Western blot, immunocytochemistry) (Shin *et al.*, 2004).

Two important wound-healing-associated proteins – heat shock protein 47 (HSP47), fibronectin (FN1) and its oncofetal splice variant – were determined to be overexpressed in superficial fibromatosis primary tissues and cell cultures as compared to reference normal tissues and cultures (Western blot, immunocytochemistry) (Howard *et al.*, 2004).

The gene expression in 6 primary cell cultures derived from palmar superficial fibromatoses was compared with the one in 6 normal palmar fascia fibroblast cell cultures using 2 different microarray platforms (GE Code LinkTM and IlluminaTM). Among the 11 genes detected to be downregulated in primary cultures on both

platforms, the ECM componets type XV collagen, proteoglycan 4 (PRG4) and fibulin-1 (FBLN1) could be found. PRG4 has also been implicated in the regulation of proliferation (Satish *et al.*, 2008).

Bioinformatic analytical techniques on existing, published gene expression data were employed in another study to identify candidate genes differentiating superficial fibromatoses from normal reference tissue. The following six genes, reported to be overexpressed in diseased tissues, could be found: a disintegrin and metalloproteinase domain 12 (ADAM12), aldehyde dehydrogenase 1 (ALDH1), iroquois homeobox protein 6 (IRX6), proteoglycan 4 (PRG4), tenascin C (TNC) and periostin (POSTN). The expression of these 6 genes was analyzed in tissues and corresponding cell cultures of superficial fibromatoses as compared to normal fascia (real-time RT-PCR). In tissues, the overexpression of ADAM12, IRX6, POSTN and TNC could be confirmed. In contrast, these genes were not differentially expressed in the corresponding cell cultures (Shih *et al.*, 2009).

2.3. Aim of the study

Agilent microarray and real-time RT-PCR analyses described in *Chapter I, Section 4.2.* revealed an overexpression of WNT5A in aggressive fibromatosis as compared to superficial fibromatosis and reference fibrous tissue. Functional cell culture experiments should bring light into the impact of WNT5A on cell signalling pathways, proliferation and invasive behaviour of fibromatosis tumor cells (*Chapter III*). Therefore, the aim of the experiments described in this chapter was the establishment and characterization of primary tumor cell cultures derived from fibromatoses. In addition, their endogenous expression and secretion of WNT5A should be analyzed.

3. Materials and methods

3.1. Techniques applied for the establishment of primary tumor cell cultures derived from fibromatoses

The collection and characterization of tissues is described in *Chapter I, Section 3.1.*

1) Outgrowth of cells from small tissue pieces
2) Pre-treatment of small tissue pieces with collagenase to obtain a single cell suspension

3.1.1. Outgrowth of cells from small tissue pieces

Biopsies of 7 aggressive fibromatoses (Aggr1-7) and 5 superficial fibromatoses (Super1-5) were cut with a razor blade in a petri-dish containing Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA) and 2.8 µg/ml Amphotericin B (Sigma-Aldrich, Saint Louis, MI) into small, ~ 1 mm^3 pieces, washed several times in the same solution, and then transferred into cell culture flasks. A limited amount of cell culture growth medium (*Section 3.5.*) was added to cover the tissue pieces, but allowing them to adhere on the bottom of the flasks. After a few days of cultivation, first cells growing out of the tissue pieces could be observed. As soon as the outgrowing cells reached a certain density, the tissue pieces were transferred into other flasks, whereas the cells were trypsinized (1xTrypsin-EDTA, Invitrogen, Carlsbad, CA) and splitted into new cell culture flasks.

3.1.2. Pre-treatment of small tissue pieces with collagenase

Primary tissues were cut with a razor blade in a petri-dish containing Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA) and 2.8 µg/ml Amphotericin B (Sigma-Aldrich, Saint Louis, MI) into pieces as small as possible. They were incubated in 1.5 ml PBS-Solution 'nach Kreis' (8 g NaCl, 0.2 g KCl, 1.435 g Na_2HPO_4, 0.2g $KHPO_4$ per liter H_2O), containing 0.25 g/l $CaCl_2$ and 2 mg/ml Collagenase A (Roche, Basel, Switzerland) on a overhead shaker at 37°C for 2-12 hours, depending of the amount of tissue and the content and composition of the extracellular matrix (ECM) to be degraded. The resulting cell suspension was pelleted through a centrifugation step (160 g, 3 minutes) and resuspended into growth medium (*Section 3.5.*) for further cultivation.

3.2. Mutation analysis

β-catenin sequence analysis was performed on a 3130x Genetic Analyzer (Applied Biosystems, Norwalk, CT) at the Laboratory for Molecular Diagnostics, University Hospital, Zürich (Prof. Zimmermann) using the following primers: forward: CATTTCCAATCTACTAATGCT, reverse: CTGCATTCTGACTTTCAGTAA (Tejpar *et al.*, 1999). APC sequence analysis was done by the Research Group Human Genetics at the Center for Biomedicine DKBW, University of Basel (Prof. Heinimann).

3.3. Digital pictures

Pictures of cell cultures were taken on the Axiovert 40 CFL microscope (Zeiss, Jena, Germany) at a magnification of 10, using the digital Powershot A650 IS camera (Canon, Tokyo, Japan) at a zoom of 3.8.

3.4. Cell cultures of normal adult human dermal fibroblasts

The cell culture of normal human dermal fibroblasts (NHDF) was purchased from PromoCell (Heidelberg, Germany). The normal human dermal fibroblast cell cultures HDF15 and HDF21 were established from normal adult human skin using the technologies described above.

3.5. Growth medium

All cell cultures were grown in endothelial cell medium 2 (EGM2) basal medium supplemented with insulin-like growth factor (IGF), fibroblast growth factor (FGF) and ascorbic acid (Lonza, Basel, Switzerland), 20% fetal calf serum (FCS), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 2.8 µg/ml Amphotericin B (Sigma-Aldrich, Saint Louis, MI) in a cell culture incubator at 37°C, 5% CO_2.

3.6. Cytoplasmic protein extraction

The description of the method used to obtain cytoplasmic protein extracts from cell cultures can be found in *Chapter III, Section 3.1.2.*

3.7. SDS-PAGE and Western blot

Details about this technique to analyze the synthesis of WNT5A in different cell cultures using cytoplasmic protein extracts can be obtained in *Chapter III, Section 3.2.*

3.8. Immunoprecipitation of WNT5A and subsequent SDS-PAGE / Western blot

Immunoprecipitation of secreted WNT5A in cell culture supernatants of NHDF, HDF15, HDF21, Aggr2, Aggr4, Aggr7 and Aggr6 was performed according to the technical bulletin of the Protein G Immunoprecipitation Kit (Sigma-Aldrich, Saint Louis, MI). WNT5A was precipitated in 600 µl of conditioned medium (3 day culture) using 5 µg of WNT5A-antibody (R&D Systems, Minneapolis, USA). Equal volumes of precipitated WNT5A (12 µl) were loaded on denaturing gels and resolved using SDS-PAGE as described in *Chapter III, Section 3.2.* Afterwards, Western blotting was performed according to the technical manual of the One-step Complete IP-Western Kit (GeneScript Corporation, Piscataway, NJ), to reduce the detection of the WNT5A-antibody used for Immunoprecipitation, since the same antibody was applied for the Western blot. After exposure of the membrane to a Hyperfilm ECL high performance

chemiluminescence film (GE Healthcare, Waukesha, WI), the film was processed as described in *Chapter III, Section 3.2.*

3.9. Agilent 60mer-oligo microarrays

For the analysis of RNA samples derived from tissue, details about this technique can be obtained from *Chapter I*, *Section 3.2.*

For the analysis of RNA samples derived from cell cultures NHDF, HDF15, HDF21, Aggr2, Aggr4, Aggr6 and Aggr7, the day before the experiment, cells were splitted into 25 cm^2 cell culture flasks at a density to reach ~80-90% confluency over night. Then, after washing in Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS), cells were trypsinized (1xTrypsin-EDTA, Invitrogen, Carlsbad, CA), centrifuged (160 g, 3 minutes) and the pelleted cells (~$1x10^6$) resuspended in 1 ml of TriReagent (Molecular Research Center, Inc., Cincinnati, OH) for subsequent total RNA extraction according to the protocol supplied by the company. Resulting total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Agilent Technologies, Santa Clara, CA). RNA quality control was performed as described in *Chapter I, Section 3.2.2.* Equal amounts of total RNA derived from cell cultures NHDF, HDF15 and HDF21 were pooled to generate a normal human dermal fibroblast RNA pool. The samples were single color (Cy3)-labelled, hybridized, and the resulting data processed according to the descriptions in *Chapter I, Section 3.2.4.*

4. Results

4.1. Mutation analysis of the established primary cell cultures

Figure 29 summarizes the results obtained from the β-catenin- and APC-mutation analyses to verify the tumoral origin of the different established primary cell cultures.

Aggr1 is derived from a Familial Adenomatous Polyposis (FAP) patient, thus its primary tumor is characterized by a biallelic inactivation of the APC gene. APC-mutation analysis revealed a germline mutation in one allele, but no somatic mutation in the second, indicating that an epigenetic promoter hypermethylation inactivated the second allele in this tumor. Accordingly, only the monoallelic germline APC-mutation was found in the corresponding cell culture. Therefore, the tumoral origin of the cell culture Aggr1 remains uncertain.

All other cell cultures from aggressive fibromatoses (Aggr2-7) are derived from sporadic cases. Aggr2-6 tumor tissues are characterized by a monoallelic β-catenin mutation at codon 45 (S45F) or codon 41 (T41A), whereas for Aggr7, no β-catenin mutation could be detected. In the corresponding cell cultures, a congruent mutation could only be found in Aggr6, proving its tumoral origin. Aggr2-5 did not show the β-catenin mutation their original tumors are characterized by. Therefore, they represent cultures of normal fibroblasts. The tumoral origin of Aggr7 remains uncertain, because its primary tumor lacks any β-catenin mutation.

Since tumor tissues of superficial fibromatoses are not affected by genetic alterations in the β-catenin- or APC-gene, their tumoral origin could not be determined.

	Mutation status (first allele / second allele)	
	Tumor	***Primary cell culture***
Aggr1 (FAP-assoc.)	APC (mut/?)	APC (mut/?)
Aggr2 (sporadic)	β-catenin (S45F/WT)	no β-catenin mutation
Aggr3 (sporadic)	β-catenin (S45F/WT)	no β-catenin mutation
Aggr4 (sporadic)	β-catenin (T41A/WT)	no β-catenin mutation
Aggr5 (sporadic)	β-catenin (T41A/WT)	no β-catenin mutation
Aggr6 (sporadic)	**β-catenin (S45F/WT)**	**β-catenin (S45F/WT)**
Aggr7 (sporadic)	no β-catenin mutation	no β-catenin mutation
Super1	no β-catenin mutation	no β-catenin mutation
Super2	no β-catenin mutation	no β-catenin mutation
Super3	no β-catenin mutation	no β-catenin mutation
Super4	no β-catenin mutation	no β-catenin mutation
Super5	no β-catenin mutation	no β-catenin mutation
	color code:	Tumoral origin of cell culture proven
		Tumoral origin of cell culture uncertain
		Tumoral origin of cell culture excluded

Figure 29: Mutation status of the different established cell cultures and their tumor tissues of origin

In order to verify that the initially proven tumor cell culture Aggr6 does not get overgrown by tumor-associated normal fibroblasts during repeated passaging in culture, the β-catenin sequencing was repeated after its passage 4, 6, 10, 14 and 21. The tumoral origin could be proven in each of the samples analyzed.

4.2. Growth characteristics of the established primary cell cultures

A prerequisite of cell growth is the addition of 20% FCS, IGF, FGF and ascorbic acid to the endothelial cell medium 2 (EGM2) basal medium.

After 23 passages, cells go into senescence. This stadium is characterized by the cessation of proliferation, an enormous expansion of the cell body's surface and the development of stress fibres (*Figure 30*: *b*) (p23) vs. *a*) (p8)). Therefore, the cells do not represent an immortalized cell line.

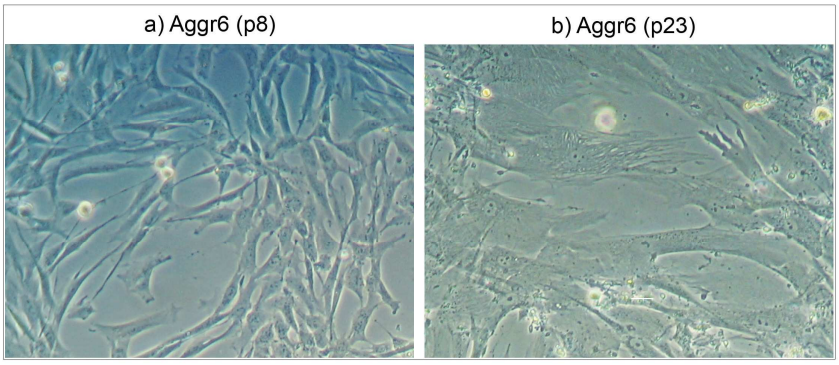

Figure 30: Pictures of Aggr6 after different numbers of passages in culture. *a*) 8 passages (p8) *b*) 23 passages (p23). Pictures were done at the same magnification.

4.3. Analysis of the endogenous WNT5A-expression

The endogenous expression of WNT5A was measured in tissues of aggressive fibromatoses on the mRNA level using Agilent microarray analysis and in the corresponding primary cell cultures on the mRNA level (Agilent microarray) as well as on the protein level using Western blot analyses on cytoplasmic protein extracts and cell cultures supernatants by immunoprecipitation. For comparison, the reference fibrous tissue RNA pool from dense connective tissues and the three cell cultures of normal human dermal fibroblasts NHDF, HDF15 and HDF21 were used accordingly. *Figure 31 a)* summarizes the results of all different analyses: WNT5A RNA expression levels (Agilent microarray), cytoplasmic WNT5A content (Western blot, *Figure 31 b)*) and WNT5A content in cell culture supernatants (immunoprecipitation, *Figure 31c)*).

WNT5A is highly overexpressed in tissues of aggressive fibromatoses as compared to reference fibrous tissues (*Figure 31 a)*, red columns 5,9,13,17 vs. red column 1). This confirms the results obtained by comparing pools of aggressive fibromatoses and reference fibrous tissues (*Chapter I*, S*ection 4.2.*).

Cell cultures of normal fibroblasts express more (Aggr4; blue columns 10,11,12) or similar amounts (Aggr2; blue columns 6,7,8) of WNT5A in comparison to the sole tumor cell culture Aggr6 (blue columns 18,19,20), whereas Aggr7 (blue columns 14,15,16) expresses less, comparable to the level expressed in the reference dermal fibroblasts (blue columns 2,3,4). These differences can be observed on the mRNA-level (blue columns 2,6,10,14,18), on the protein level in the cytoplasm (blue columns 3,7,11,15,19), as well as on the level of WNT5A secreted into the supernatant (blue columns 4,8,12,16,20).

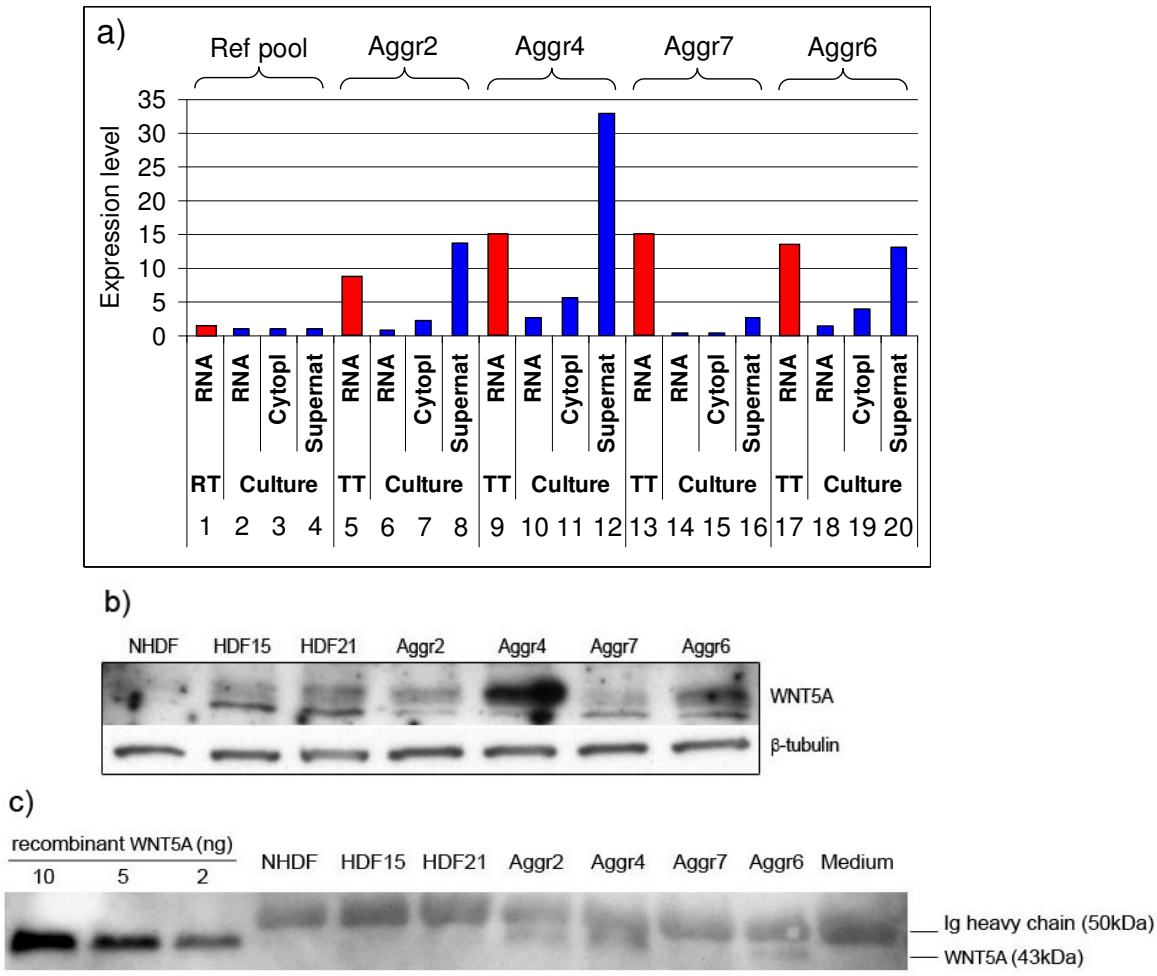

Figure 31: Endogenous WNT5A-expression in tumor tissues of aggressive fibromatoses as compared to reference fibrous tissues and in corresponding primary cell cultures.

a) WNT5A-expression measured on the mRNA level (RNA) using Agilent microarrays and on the protein level on Western blots using cytoplasmic protein extracts (Cytopl) and cell culture supernatants (Supernat). Aggr2, Aggr4, Aggr7, Aggr6 tumor tissues (red bars TT) and the reference fibrous tissue pool (red bar RT) were analyzed using Agilent microarrays, whereas the corresponding cell cultures (blue bars Culture) were examined using both Agilent microarrays and Western blots. The three values for the Culture Ref pool represent the means of the values obtained analyzing the single cell cultures of normal human dermal fibroblasts NHDF, HDF15 and HDF21.

b) WNT5A-expression measured on the protein level on a Western blot using cytoplasmic protein extracts of cell cultures of normal human dermal fibroblasts NHDF, HDF15 and HDF21 and of aggressive fibromatoses Aggr2, Aggr4, Aggr7, and Aggr6. The intensity of the bands was quantified and the resulting values integrated as columns (Cytopl) into *Figure 31 a).* β-tubulin: loading control.

c) WNT5A-expression measured on the protein level on a Western blot using immunoprecipitated supernatants of cell cultures of normal human dermal fibroblasts NHDF, HDF15 and HDF21 and of aggressive fibromatoses Aggr2, Aggr4, Aggr7, and Aggr6. The intensity of the bands was quantified and the resulting values integrated as columns (Supernat) into *Figure 31 a).* Different amounts of recombinant WNT5A were added to estimate the amount of endogenously produced WNT5A secreted by the cell cultures. Ig heavy chain: Immunoglobulin heavy chain of the WNT5A-antibody used to immunoprecipitate WNT5A.

In several experiments described in *Chapter III*, primary cell cultures of aggressive fibromatoses and reference normal fibroblasts will be treated with recombinant WNT5A to analyze its impact on cellular signalling pathways and behaviour. The aim of the addition of three different amounts of recombinant WNT5A on the gel analyzing WNT5A-immunoprecipitated cell culture supernatants (*Figure 31c*) was it therefore to approximately quantify the amount of WNT5A secreted by these cell cultures. This allows then to estimate the proportion of secreted WNT5A in comparison to the added recombinant WNT5A. The calculations yielded the following result: The cell culture Aggr4, producing the highest amount of WNT5A (*Figure 31c*), secrets ~ 60 ng WNT5A during 72 hours, per 1.5 millions of cells into 36 ml of medium. In a typical experiment presented in *Chapter III*, 150 ng/ml recombinant WNT5A was added to ~1 million of cells in 4 ml of medium, thus an amount (600ng), that is 15 times higher then the WNT5A secreted by 1 million of Aggr4 cells during 72 hours (40ng). Aggr6 and the other cell cultures analyzed secrete even less.

5. Discussion

5.1. Summary of the results

Of the five cell cultures (Aggr2-6) being derived from β-catenin mutated aggressive fibromatosis tumors, only one (Aggr6) undoubtedly proved to represent a tumor cell culture, whereas the others certainly are cultures of normal fibroblasts. Concerning Aggr1, Aggr7 and all the superficial fibromatosis cell cultures, their tumoral origin could not be determined using mutation analyses due to a missing mutation in the second APC-allele (Aggr1), or due to missing mutations in the β-catenin gene (Aggr7, all Supers) in the tumor tissues.

The established primary cell cultures do not represent immortalized cell lines, since they go into the stadium of senescence after 23 passages.

Aggressive fibromatosis tumor tissues highly overexpress WNT5A as compared to reference fibrous tissues. In contrast, the sole tumor cell culture Aggr6 is not characterized by an increased expression or secretion of WNT5A as compared to cell cultures of normal fibroblasts. There is a pronounced correlation between WNT5A mRNA synthesis, WNT5A protein expression and WNT5A protein secretion in the cell cultures analyzed. The amount of endogenously secreted WNT5A by these cell cultures represents just a small portion of the amount of recombinant WNT5A usually added to the cells in a typical experiment described in *Chapter III*.

5.2. Published studies using primary cultures of aggressive and superficial fibromatoses

Several papers published in the literature report about the use of primary tumor cell cultures derived from fibromatoses in functional studies (*Section 2.2.*).

The papers dealing with primary cell cultures of aggressive fibromatoses are summarized in *Figure 32*. It is striking that the mutation status of the tumor tissues is in most of the cases properly annotated (*column 2*), whereas the one for the corresponding primary cell cultures is always missing (*column 3*). Therefore, the formal proof that these cell cultures represent primary tumor cell cultures and not cultures of tumor-associated fibroblasts is lacking in all these studies. Instead, the authors try to prove the tumoral origin of their cell cultures in the following ways:

In one study, the authors used instead an immunohistochemical approach, showing that an antibody raised against the C-terminal part of APC detects this protein in cultures of normal fibroblasts, but not of aggressive fibromatoses, due to their biallelic C-terminal truncating mutations in the gene coding for this protein (Li *et al.*, 1998).

In addition, the same authors report about an accumulation of β-catenin in these tumor cells (Western blot, whole cell protein extracts) that can be repressed by an exogenous expression of wildtype APC (Li *et al.*, 1998). Two other papers report about an enhanced accumulation of nuclear β-catenin in the cell cultures of aggressive fibromatoses as compared to cultures of control fibroblasts, using immunhistochemistry (Amini Nik *et al.*, 2005; Denys *et al.*, 2004b). A similar

argument for the tumoral origin of their cell cultures provide the authors of another report, showing an increased nuclear β-catenin staining in tumoral cell cultures using confocal immunofluorescence microscopy (Tejpar *et al.*, 1999).

The authors of three studies argue for a tumoral origin of their cell cultures used by describing their increased TCF-LEF-dependent transcriptional activity (Luciferase-reporter assay) as compared to the one of normal fibroblasts (Amini Nik *et al.*, 2005; Denys *et al.*, 2004b; Tejpar *et al.*, 2001).

In two further papers, the authors do not tell anything about the 'tumoral origin' of their cell cultures (Denys *et al.*, 2004a; Locci *et al.*, 2001).

Thus, since in all these studies mutation analyses to prove the tumoral origin of the primary cell cultures used are missing, it is at least questionable whether all the reported cultures in the literature truly represent tumor cell cultures.

Aggressive fibromatosis tumor tissue		**Primary cell culture**	**Paper**
Number and type	**Mutation status**	**Prove of tumoral origin**	
4 x FAP-assoc.	APC: biallelic	Antibody C-terminus APC (IHC) Increased β-catenin (Western, whole cell)	Li *et al*., 1998
1 x FAP-assoc.	APC: biallelic	Increased nuclear β-catenin (Confocal microscopy)	Tejpar *et al*., 1999
3 x Sporacic	β-catenin: monoallelic		
2 x Sporadic	β-catenin: no mutation		
? x FAP-assoc.	APC: biallelic	No prove provided	Locci *et al*., 2001
2 x FAP-assoc.	APC: biallelic	Increased TCF-LEF-reporter assay activity	Tejpar *et al.*, 2001
6 x Sporadic	β-catenin: monoallelic		
2 x Sporadic	β-catenin: no mutation		
2 x FAP-assoc.	APC: biallelic	Increased nuclear β-catenin accum. (IHC) Increased TCF-LEF-reporter assay activity	Denys *et al*., 2004b
2 x Sporadic	β-catenin: monoallelic		
7 x	not described	No prove provided	Denys *et al.*, 2004a
2 x FAP-assoc.	APC: biallelic	Increased nuclear β-catenin accum. (IHC) Increased TCF-LEF-reporter assay activity	Amini Nik *et al*., 2005
3 x Sporacic	β-catenin: monoallelic		

Figure 32: Studies published in the literature describing the use of primary tumor cell cultures of aggressive fibromatoses and the approach to prove their tumoral origin. Number and type: number of sporadic or familial adenomatous polyposis (FAP)-associated aggressive fibromatosis tumor tissues taken into culture.

The situation is even more questionable, when looking at the publications reporting the use of primary tumor cell cultures derived from superficial fibromatoses (*Section 2.2.2.*). In those papers, the authors do not tell anything about a characterization of their cell cultures used.

5.3. Endogenous WNT5A expression

The primary cell culture Aggr6 represents the only established primary aggressive fibromatosis tumor cell culture. Therefore, a similar overexpression of WNT5A in this verified tumor cell culture would be expected in comparison to all the other cultures of normal fibroblasts, if the differences observed on the tissue level are transferable into primary cell cultures. Our data demonstrate that such a correlation between tissues and cell cultures does not exist for the expression of WNT5A. Two explanations may come up with this result.

First, both tumor cells and tumor-associated fibroblasts are responsible for the observed overexpression of WNT5A in tissues of aggressive fibromatoses. An immunohistochemistry (IHC) and in-situ hybridization (ISH) approach was attempted

to help to clarify this issue. But due to a missing specificity of the WNT5A-antibody (IHC) and an unsatisfying reproducibility of the ISH-results, this attempt had to be abandoned.

The second explanation for the missing correlation between WNT5A-expression in tumor tissues and cell cultures is the completely modified microenvironment tumor cells are faced to in artificial in vitro cell cultures as compared to the highly complex cell-cell- and cell-extracellular matrix (ECM)-interactions tumor cells are involved in in vivo. Missing influences of neighbouring cells and/or ECM-proteins may therefore be responsible for the observed reduced WNT5A-expression in the tumor cell culture Aggr6. In fact, several studies published in the literature claim that the expression of WNT5A in different cell cultures is inducible after the addition of certain stimuli.

Human umbilical venous endothelial cells (HUVEC) react to inflammatory mediators with an increased expression of WNT5A, leading to their enhanced proliferation and migration (Cheng *et al.*, 2008). Stimulation of human T-lymphocyte cultures with the chemokine CXCL12 induces their secretion of WNT5A that in turn exerts a positive autocrine effect on their migratory behaviour (Ghosh *et al.*, 2009). Cell cultures of human macrophages respond to the addition of LPS or interferon gamma with an enhanced expression of WNT5A that induces their secretion of proinflammatory cytokines (Pereira *et al.*, 2008). Rat neonatal heart myocyte cell cultures show an enhanced expression of WNT5A after the interleukin-6-dependent activation of the transcription factor STAT3. This leads to a stabilization of N-cadherin and an enhanced cell adhesion (Fujio *et al.*, 2004). In cell cultures of human papillary thyroid cancer, pancreatic cancer and cutaneous melanoma, the expression of WNT5A is dependent on the activation of the Toll-like receptor 3 (TLR3) that in turn activates STAT3 through the upregulation of interleukin-6. Through this mechanism, the proliferation and migration of those tumor cells gets enhanced (McCall *et al.*, 2007; Schwartz *et al.*, 2009). In human pancreatic cancer cell cultures, TGFβ was shown to activate the transcription factor CUTL1 that upregulates the transcription of WNT5A, leading to increased migration, invasion, proliferation, and expression of marker genes associated with epithelial-mesenchymal transition (EMT) (Ripka *et al.*, 2007). In human cutaneous melanoma cell cultures, the expression of WNT5A gets enhanced after the addition of heparan sulfate proteoglycans (HSPGs), components of the ECM that induce in this way the invasive capacity of those cells (O'Connell *et al.*, 2009).

On the other hand, genetic alterations of the WNT5A gene, such as amplifications, leading to an enhanced expression of WNT5A in tumor cells, have not been described in the literature up to date.

5.4. Possible approaches to improve the rate of success for the establishment of primary tumor cell cultures

The experiments done to establish primary cell cultures of aggressive and superficial fibromatoses impressively demonstrated that this approach is a tricky task. Of the five cell cultures (Aggr2-6) being derived from β-catenin-mutated aggressive fibromatosis tumors, only one undoubtedly proved to represent a tumor cell culture, whereas the others certainly are cultures of normal fibroblasts. Concerning Aggr1, Aggr7 and all the superficial fibromatosis cell cultures, their tumoral origin could not be determined

using mutation analyses due to a missing mutation in the second APC-allele (Aggr1), or due to missing mutations in the β-catenin gene (Aggr7, all Supers) in the tumor tissues.

The question therefore raises, whether there may be some technical improvements feasible to specifically enrich the number of fibromatosis tumor cells before taking them into culture in order to increase the likelihood for establishing a primary tumor cell culture.

One approach would be to transfect the cells with a TCF-reporter construct and sort for the positive cells using fluorescence-activated cell sorting (FACS) analysis, since a hallmark of all fibromatoses is an accumulation of β-catenin in the nucleus and therefore an increased TCF-dependent transcription (Alman *et al.*, 1997a; Montgomery *et al.*, 2001; Tejpar *et al.*, 1999; Varallo *et al.*, 2003). Two aspects impair the usefulness of this approach. First of all, our study revealed that tumor-associated fibroblasts of aggressive fibromatoses are characterized by an enhanced – although reduced in comparison to tumor cells – activity of TCF-dependent transcription as well (*Chapter III, Section 4.2.1.*). Therefore, the setting of the threshold-level for the sorting process may be difficult. Secondly, there is no study published so far describing the activity of TCF-dependent transcription in tumor cell cultures of fibromatoses lacking any β-catenin or APC-mutation. It is unknown whether these cells retain their constitutive activation when taken into culture, since the dogma of constitutive activation of TCF-LEF-dependent transcription in all fibromatoses, independent of any APC- or β-catenin mutation background, is derived from β-catenin immunohistochemical analyses on tissue sections. Therefore, whereas such a FACS approach may be appropriate for tumors carrying APC- or β-catenin-mutations, it may not be feasible for superficial fibromatoses and aggressive fibromatoses without β-catenin/APC-mutations.

Another approach would be to sort the cells prior cultivation using a cell membrane marker that is specifically overexpressed in fibromatoses tumor cells as compared to tumor-associated normal fibroblast. Unfortunately, such a marker has not been found, yet.

Aggressive and superficial fibromatoses are characterized by their capability to invade into neighbouring tissues (*Chapter I, Section 2.1.*). Primary tumor cells may show an enhanced invasive growth in an in vitro invasion assay. Selection and further culturing of the first cells being able to invade through the ECM-barrier in such an assay may therefore be a possible approach to specifically enrich tumor cells.

A selection for those cells that show a higher proliferation rate may be another possibility.

Chapter III
The impact of WNT5A on cell signalling pathways and biological behaviour of aggressive fibromatosis tumor cells

1. General introduction

In this chapter, the focus is laid on the analysis of WNT5A's impact on cell signalling pathways and biological behaviour of aggressive fibromatosis tumor cells. Since WNT5A is known to activate both canonical and non-canonical Wnt pathways, the latter will be introduced in the following general introduction. A comprehensive description of the canonical Wnt pathway can be found in *Chapter I, Section 1.1.* Subsequently, published studies analyzing the role of WNT5A in the pathogenesis of cancer will be reviewed.

1.1. The non-canonical Wnt pathways

In this section, the non-canonical – meaning β-catenin-independent – Wnt signalling pathways should be introduced on the basis of their components. In addition, their crosstalks with the canonical Wnt signalling pathway should be demonstrated.

1.1.1. The Wnt-Ca^{2+} signalling pathway

The idea that there are multiple Wnt signalling pathways came up with experiments in Xenopus embryos and mouse C57MG mammary epithelial cells showing that vertebrate Wnts can be divided into two groups according to their biological activities. Ectopic expression of the first group of Wnts (including WNT1, WNT3A, WNT8 and WNT8B) induces a secondary axis in Xenopus embryos (Du *et al.*, 1995) and morphologically transforms C57MG mammary epithelial cells (Wong *et al.*, 1994) through the activation of the canonical Wnt pathway. In contrast, the expression of the second group of Wnts (WNT4, WNT5A, WNT11) neither induces a second axis in Xenopus, nor transforms C57MG cells. However, these Wnts are biologically active, since they are important for convergent extension movements (directed migrations of cells driving the mediolateral convergence and anteroposterior extension of the body) during Xenopus embryogenesis (Du *et al.*, 1995; Moon *et al.*, 1993; Torres *et al.*, 1996).

Further experiments, mainly done using WNT5A, supported the hypothesis that there is more than one signalling pathway induced by the Wnts. In contrast to the first group of Wnts that transform C57MG cells after ectopic expression, loss of function of WNT5A by antisense RNA is also transforming (Olson and Gibo, 1998). WNT5A induces a secondary axis in Xenopus via the canonical Wnt pathway when coexpressed with the Frizzled (Fzd) receptors Fzd4, Fzd5, Fzd7 or Fzd8 (Deardorff *et al.*, 1998; He *et al.*, 1997; Itoh *et al.*, 1998; Umbhauer *et al.*, 2000). WNT5A blocks the ability of WNT8 to induce a secondary axis (Torres *et al.*, 1996). These experiments led to the assumption, that the different cellular responses elicited by the various Wnts in Xenopus and C57MG cells are derived from multiple pathways induced by the signalling specificities of each single Wnt-Fzd receptor interaction.

This point of view got further support by a recent study in human embryonic kidney cells, demonstrating that WNT5A activates the canonical Wnt pathway when coexpressed with Fzd4 and LRP5, but inhibits the same when coexpressed with ROR2 (Mikels and Nusse, 2006).

Subsequent experiments were dedicated to elucidate the details of the so far unknown Wnt signaling pathway (*Figure 33*). WNT5A, but not the axis-inducing WNT8, triggers an intracellular Ca^{2+} release (Slusarski *et al.*, 1997b). Ectopic expression of a serotonin receptor, known for its induction of intracellular Ca^{2+} release, blocks the ability of WNT8 to induce a secondary axis, thus phenocopies the effect of WNT5A, showing that WNT5A inhibits the canonical Wnt pathway in a Ca^{2+}-dependent manner (Slusarski *et al.*, 1997b). The intracellular Ca^{2+} release induced by WNT5A is dependent upon heterotrimeric G proteins, since this effect can be blocked by the addition of the specific G protein inhibitor Pertussis toxin. Heterotrimeric G proteins are so named because of their three distinct subunits $G\alpha$, $G\beta$, $G\gamma$, and because the activity of $G\alpha$ is dependent on the binding of guanosine triphosphate (GTP). In its inactive state, $G\alpha$ binds guanosine diphosphate (GDP). In addition, an inositol monophosphatase inhibitor blocks the intracellular Ca^{2+} efflux as well, an effect that can be avoided by concomitant treatment with myoinositol (Slusarski *et al.*, 1997a), indicating that $G\alpha$ activates phospholipase C β (PLCβ), that in turn induces the intracellular Ca^{2+} release through the cleavage of phosphatidylinositol diphosphate (PIP_2) to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). Thus, WNT5A inhibits the canonical Wnt pathway by the activation of the phosphatidylinositol pathway in a G protein-dependent manner leading to an intracellular Ca^{2+} efflux. Subsequently, it was shown that WNT5A activates the downstream, Ca^{2+}-sensitive enzymes calcium-calmodulin-dependent protein kinase II (CamKII) (Kuhl *et al.*, 2000a) and protein kinase C (PKC) (Sheldahl *et al.*, 1999). In contrast, WNT8, that activates the canonical Wnt pathway, does not activate either CamKII or PKC. After these findings, the newly detected Wnt pathway was termed Wnt-Ca^{2+} pathway (Kuhl *et al.*, 2000b). Further components of Wnt-Ca^{2+}-pathway could also be found, namely the transcription factor nuclear factor of activated T cells (NFAT) that gets dephosphorylated and activated through the calmodulin-dependent phosphatase calcineurin (PP2B) (Saneyoshi *et al.*, 2002) and Dishevelled (Dsh), that is also involved in the canonical Wnt pathway (Sheldahl *et al.*, 2003).

In other studies, the components of the canonical Wnt and Wnt-Ca^{2+} pathway were determined that are responsible for the observed crosstalk between these two pathways. It turned out that the canonical Wnt pathway is absolutely necessary for convergent extension movements during Xenopus gastrulation, but that the Wnt-Ca^{2+} pathway exerts an important modulating effect on it, by inhibiting Dsh through PKC and LEF via CamKII and therefore inhibiting the activity of the canonical Wnt pathway (Kuhl *et al.*, 2001). Working with human embryonic kidney cells, the authors of another study report that the CamKII induced inhibition of the canonical Wnt pathway is dependent upon a downstream activation of the MAPKs TAK1 and NLK (Ishitani *et al.*, 2003).

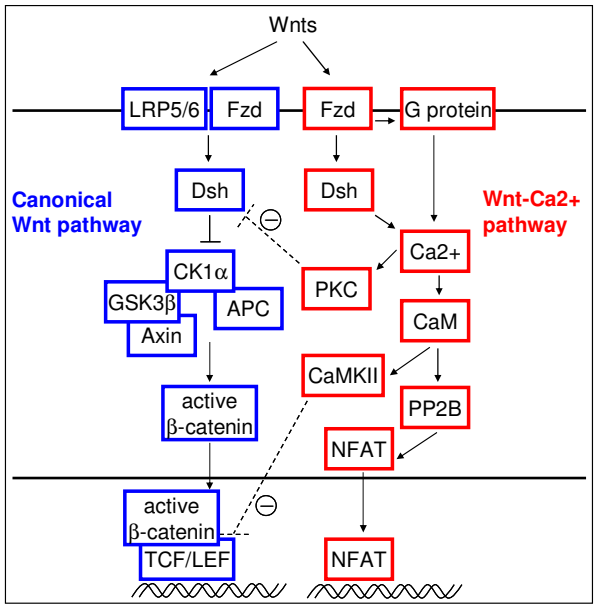

Figure 33: The components of the canonical Wnt and the Wnt-Ca^{2+} pathway and their crosstalk

1.1.2. The Wnt-PCP signalling pathway

A series of experiments indicated that the observed modulation of convergent extension movements in Xenopus embryogenesis is not solely dependent upon the Wnt-Ca^{2+} signalling pathway, but at least in part upon another non-canonical Wnt pathway that shows similarities to the planar cell polarity (PCP) pathway in Drosophila and is therefore termed Wnt-PCP pathway. In Drosophila, the PCP pathway is needed to control the orientation of hairs, bristles and ommatidia during embryogenesis.

In the following review of the literature, the components of the Wnt-PCP pathway will briefly be introduced (*Figure 34*). Whereas the DIX-domain of Dsh is needed to activate the canonical Wnt pathway, its DEP-domain is absolutely required for the induction of both Drosophila PCP and vertebrate Wnt-PCP pathway (convergent extension movements) (Axelrod *et al.*, 1998; Boutros *et al.*, 1998; Heisenberg *et al.*, 2000; Tada and Smith, 2000). A Dsh-construct lacking the DIX-domain – thus activating the Wnt-PCP pathway but not the canonical Wnt pathway – was shown to be a strong inducer of the Wnt-Ca^{2+} pathway as well (Sheldahl *et al.*, 2003). These findings indicate that the canonical Wnt pathway diverges from the non-canonical pathways at the level of Dsh, whereas the Wnt-Ca^{2+} pathway diverges from the Wnt-PCP pathway more downstream (Veeman *et al.*, 2003). Further mediators of the Drosophila PCP and vertebrate Wnt-PCP pathway include members of the Rho family of small GTPases, namely Rho, Rac and Cdc42. In Drosophila, Rho acts downstream of Dsh (Strutt *et al.*, 1997), whereas a function for Cdc42 has also been proposed (Eaton *et al.*, 1996). Rho, Rac and Cdc42 have all been implicated in vertebrate Wnt-PCP signalling (Choi and Han, 2002; Habas *et al.*, 2003; Habas *et al.*, 2001; Penzo-Mendez *et al.*, 2003), whereupon a parallel induction of Rho and Rac by Dsh has been shown to be essential (Habas *et al.*, 2003). More downstream of the small GTPases, the c-jun-N-terminal kinase (JNK) comes into play. It is essential for the PCP pathway in Drosophila (Boutros *et al.*, 1998). Which small GTPases are responsible for JNK-activation in the vertebrate Wnt-PCP pathway is controversially

discussed, with one report stating that JNK is activated through Dsh and Rac, but not Rho or Cdc42 (Habas *et al.*, 2003), the other study showing that JNK induction requires both Rac and Cdc42 (Moriguchi *et al.*, 1999).

In addition, there's a plethora of studies reporting that WNT5A activates JNK through binding to the receptor tyrosine kinase-like orphan receptor 2 (ROR2). In a recent study describing this connection, the authors claim that the resulting modulation of convergent extension movements in Xenopus embryos should be considered as a novel non-canonical Wnt pathway, since it functions independent of Dsh, known to be essential for the Wnt-PCP pathway (Schambony and Wedlich, 2007). However, the authors of another study working with human osteosarcoma cell cultures report that WNT5A signals via ROR2 and Dsh2 to activate JNK, leading to increased invasion and migration, suggesting that ROR2 represents an alternative receptor for WNT5A to activate the Wnt-PCP pathway (Enomoto *et al.*, 2009). This view is supported by the work demonstrating that ROR2 functions as a coreceptor for Fzd receptors, similar to the LRP5/6 coreceptors, but with different downstream activation characteristics. After WNT5A-binding to ROR2, its intracellular domain gets phosphorylated by certain kinases, leading to the activation of ROR2 and the inhibition of the canonical Wnt pathway through the involvement of unknown downstream components (Winkel *et al.*, 2008). On the other hand, after binding of a 'canonical' Wnt, such as WNT1, ROR2 does not get phosphorylated and activated. In contrast, this interaction leads to a potentiation of the canonical Wnt-signalling pathway activation, probably because ROR2 supports the binding of the canonical Wnt to its cognate receptor. Thus, depending on the Wnt binding, ROR2 functions as a Fzd coreceptor to potentiate or repress canonical Wnt-signalling. The authors of another study draw the same conclusion, demonstrating that ROR2 functions as a Fzd coreceptor important to activate the Wnt-PCP pathway, but suppressing the canonical Wnt pathway (Yamamoto *et al.*, 2008). This opinion is further underlined by two studies showing that WNT5A, but not the canonical WNT3A, induces the dimerization of ROR2, leading to the activation of the tyrosine kinase SRC (Akbarzadeh *et al.*, 2008) or the serine/threonine kinases GSK3α/β (Liu *et al.*, 2008b), that in turn phosphorylate and activate the intracellular domain of ROR2. Further studies are needed to elucidate the function of ROR2 in context of Wnt-PCP signalling. However, whether it functions as a Fzd-independent or a Fzd coreceptor, whether it induces the Wnt-PCP pathway or a Dsh-independent, novel non-canonical pathway: most studies published report that it represents the relay station between Wnt and JNK. Therefore, to simplify matters, ROR2-dependent JNK-activation is going to be considered as an activated Wnt-PCP signalling pathway in the following presentation of the literature.

JNK functions as a mitogen-activated protein kinase (MAPK), phosphorylating and activating among others components of the dimeric activator protein (AP-1) transcription factor complex such as the c-jun, junB and junD. AP-1 is composed of several different heterodimeric complexes of proteins derived from the fos and jun families including c-fos, fosB, fra-1, fra-2, c-jun, junB and junD. Upon phosphorylation by JNK or other MAPKs and subsequent dimerization, the AP-1 transcription factor complexes translocate into the nucleus and activate the transcription of AP-1-dependent target genes (Eferl and Wagner, 2003; Vogt, 2001).

A vast literature describes an inhibitory crosstalk between the Wnt-PCP pathway and the canonical Wnt pathway: in Xenopus embryogenesis modulating convergent

extension movements (Schambony and Wedlich, 2007; Yamanaka *et al.*, 2002), in human osteosarcoma cell lines (Enomoto *et al.*, 2009), in human embryonic kidney cells (Mikels *et al.*, 2009; Mikels and Nusse, 2006; Winkel *et al.*, 2008; Yamamoto *et al.*, 2008) and in an axin-LacZ-canonical Wnt pathway reporter mouse model (Mikels *et al.*, 2009). Nevertheless, the identification of the exact components of these two pathways being involved in this crosstalk has not been described, yet.

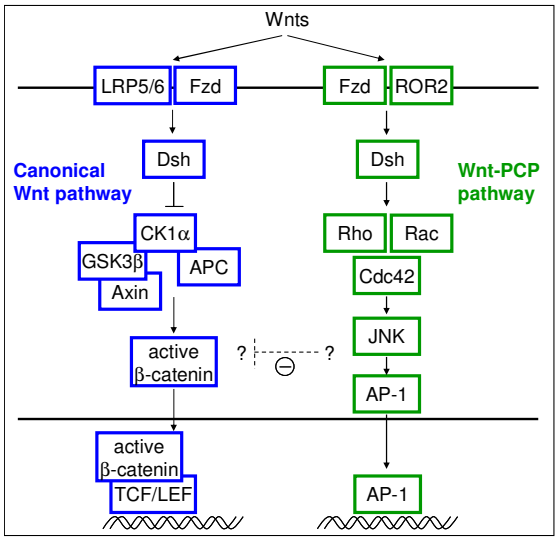

Figure 34: The components of the canonical Wnt and the Wnt-PCP pathway and their crosstalk

1.1.3. The cAMP-dependent protein kinase (PKA) pathway

The cAMP-dependent protein kinase (PKA) pathway has not been categorized as a 'real' non-canonical Wnt pathway, since only two studies involving WNT5A proved its activation through Wnts, yet (Hansen *et al.*, 2009; Torii *et al.*, 2008).

Normally, the activity of this pathway is started upon binding of ligands, such as growth factors or neurotransmitters, to G protein-coupled receptors (GPCR) (*Figure 35*). These receptors then activate a heterotrimeric G protein, so named because it consists of three distinct subunits (Gα, Gβ, Gγ), and because the activity of Gα is dependent on the binding of guanosine triphosphate (GTP). In its inactive state, Gα binds guanosine diphosphate (GDP). Upon stimulation through the GPCR, Gα switches into its active state, allowing its dissociation from the two other partners and its activation of the adenylyl cyclase (AC). The AC produces the ubiquitous intracellular messenger cyclic adenosine monophosphate (cAMP) by converting adenosine triphosphate (ATP). The major function of cAMP is activating the cAMP-dependent protein kinase (PKA), a tetramer protein consisting of two catalytic subunits and two regulatory subunits that upon cAMP binding dissociate from the catalytic subunits. The free activated PKA catalytic subunits then translocate into the nucleus and phosphorylate the cAMP response element binding protein (CREB) at serine 133, rendering it active to bind to the promoters of target genes and induce their transcription (Chiaradonna *et al.*, 2008).

Upon activation through WNT5A, PKA was reported to phosphorylate and inactivate GSK3β at serine 9, leading to the accumulation of β-catenin and its translocation into

the nucleus, therefore inducing an activating crosstalk between the PKA-pathway and the canonical Wnt-pathway (Torii *et al.*, 2008).

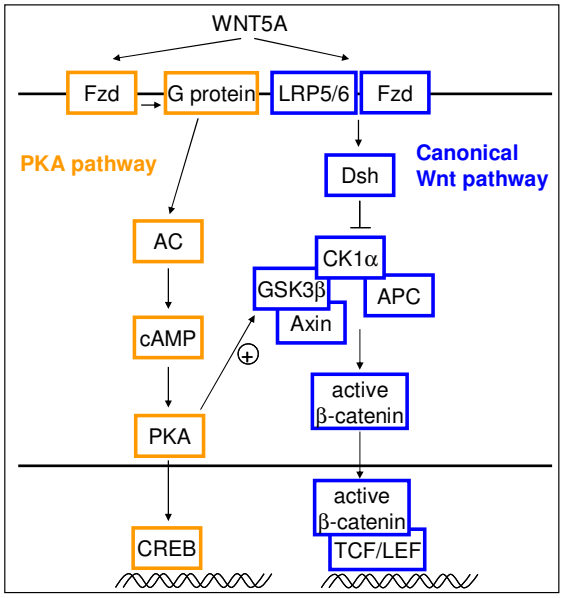

Figure 35: The components of the canonical Wnt and the PKA pathway and their crosstalk

1.1.4. The signal transducer and activator of transcription (STAT) pathway

As the PKA pathway, the STAT pathway has only recently been described to be induced by WNT5A and therefore is not considered as an established non-canonical Wnt pathway (Dissanayake *et al.*, 2008) (*Figure 36*).

The signal transducer and activator of transcription (STAT) family of transcription factors are activated by virtually every cytokine and growth factor and therefore are essential components in the immune system. Their activity is induced through tyrosine phosphorylation by ligand-activated receptor tyrosine kinases (such as epidermal growth factor receptor, EGFR) or by receptors that lack intrinsic tyrosine kinase activity but to which janus kinases (JAK) are associated (such as receptors for interferon-α or interleukin-13). The STAT family consists of seven members. After tyrosine phosphorylation, STAT dimers and heterodimers, but not monomers, are competent to bind to the promoters of target genes (Aaronson and Horvath, 2002). In the study describing an activation of the STAT pathway by WNT5A, JAK was not involved. Instead, WNT5A activated STAT3 in a PKC-dependent manner.

No crosstalk with the canonical Wnt signalling pathway was reported.

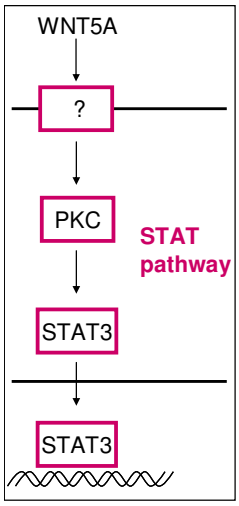

Figure 36: The components of the STAT pathway

1.2. The role of WNT5A in the pathogenesis of cancer

In the following two sections, studies published in the literature should be presented that brought WNT5A into context with the pathogenesis of a diverse array of human cancers. The first section deals with functional studies in cell cultures and animal models, whereas the second section is dedicated to the discussion of studies linking the expression of WNT5A to clinical data.

1.2.1. Functional studies in cell cultures and animal models

The results discussed in this section are summarized in *Figure 37*.

- Breast cancer

The role of WNT5A in the pathogenesis of breast cancer is controversially discussed in the literature.

In one study using the human immortalized mammary epithelial cell line HC11, WNT5A was shown to increase the proliferation rate by transactivating the receptor tyrosine kinase EGFR through the canonical Wnt pathway leading to the expression of matrix metalloproteinases (MMPs) and the resultant increase of the availability of EGFR ligands (Civenni *et al.*, 2003). This study indicates therefore that WNT5A might exert tumor-promoting capabilities in the pathogenesis of breast cancer through the activation of the canonical Wnt pathway. This finding is supported by a report stating that macrophages-derived WNT5A promotes the invasive capacity of the human breast cancer cell line MCF-7 by activating the Wnt-PCP pathway (Pukrop *et al.*, 2006).

All the other studies analyzing the role of WNT5A in the pathogenesis of breast cancer favour a tumor-suppressing function for this protein. In the first study ever that brought WNT5A into context with tumorigenesis, it was reported that the inhibition of its expression through the use of antisense RNA in mouse mammary epithelial C57MG cells leads to their transformation (Olson and Gibo, 1998). Working with the human breast cancer cell line MCF-7 that was used in the study reported before (Pukrop *et al.*, 2006), the authors came to the conclusion, that WNT5A inhibits the migration of the cells by activating the PKA pathway (Hansen *et al.*, 2009). An

inhibiting impact on both migration and invasion by WNT5A through the activation of the Wnt-Ca^{2+} pathway was reported for the human immortalized mammary epithelial cell line HB2 (Dejmek *et al.*, 2006). The remaining studies report a tumor-suppressive function of WNT5A as well, but do not demonstrate the exact signalling pathway by which WNT5A achieves its effect. An inhibiting effect on the canonical Wnt pathway was reported in mouse mammary cell cultures, leading to an increased differentiation of the cells (Roarty *et al.*, 2009). In human breast cancer cell lines, WNT5A was shown to suppress invasion by increasing intercellular adhesion (Medrek *et al.*, 2009). In estrogen receptor-α (ERα)-negative human breast cancer cell lines, the addition of WNT5A led to an increased ERα expression through a reduction of its promoter methylation. Consequently, these cells regain their responsiveness to Tamoxifen (Ford *et al.*, 2009a). Finally, there are two studies analyzing the impact of WNT5A in a mouse model: Mouse 4T1 breast cancer cells inoculated in mouse fat pad generated less metastases to lungs and liver when mice were treated with WNT5A (Safholm *et al.*, 2008). In a mouse ERα-negative breast cancer model, the injection of WNT5A led a reduction of liver and lung metastases, and an upregulation of ERα (Ford *et al.*, 2009b).

- Cutaneous melanoma

Concerning cutaneous melanoma, there is a consensus in the literature that WNT5A exerts a tumor promoting effect. Working with human cutaneous melanoma cell cultures, the authors of several studies report an activation of the Wnt-Ca^{2+} and Wnt-PCP pathway by WNT5A, leading to an increased migration and invasion of the cells (O'Connell *et al.*, 2009a; Weeraratna *et al.*, 2002; Witze *et al.*, 2008). In the same cell cultures, an activation of the STAT pathway probably through the Wnt-Ca^{2+} pathway was described, leading to a suppression of the expression of tumor-associated antigens MITF, MART-1 (Dissanayake *et al.*, 2008). Heparan sulfate proteoglycans (HSPGs) were shown to exert an expression inducing effect on WNT5A, leading to an increased invasive behaviour of cultured human cutaneous melanoma cells (O'Connell *et al.*, 2009b). WNT5A was shown to increase the intracellular Ca^{2+} mobilization in cutaneous melanoma cell cultures, through the activation of Fzd2 and cGMP-phosphodiesterase 6 (PDE6) (Bazhin *et al.*, 2009). Additionally, a positive effect of WNT5A on proliferation and migration via an unknown signalling pathway was reported (Schwartz *et al.*, 2009). Using in vitro and in vivo metastasis assays, the authors of another study showed that the Wnt-PCP pathway (ROR2) is necessary for the WNT5A-mediated metastasis of cutaneous melanoma cells (O'Connell *et al.*, 2009c).

- Hematopoietic neoplasia

In the pathogenesis of hematopoietic neoplasia, the data in the literature favour a tumor suppressive role for WNT5A. In mouse B lymphocytes from fetal liver, WNT5A activated the Wnt-Ca^{2+} pathway with a subsequent reduction in the expression level of cyclin D1 and a diminished proliferation rate, indicating an inhibitory crosstalk between the Wnt-Ca^{2+} and the canonical Wnt pathway in these cells, initiated by WNT5A (Liang *et al.*, 2003). This study got further support by a paper showing that the WNT5A promoter is hypermethylated in all human acute lymphoblastic leukemia cell lines analyzed and that the hypermethylation in primary tissues of this disease correlates with an upregulation of cyclin D1 (Roman-Gomez *et al.*, 2007). WNT5A promoter hypermethylations were also found in cell cultures of human nasal NK/T-

cell lymphoma, leukemia and Burkitt lymphoma, further supporting the view of a tumor suppressive role for WNT5A in hematopoietic neoplasia (Ying *et al.*, 2007).

- Gastric cancer

Two studies analyzing the role of WNT5A in the formation of gastric cancer led to the conclusion that it promotes carcinogenesis of this tumor. In the first report, the authors describe that WNT5A induces cell adhesion and migration of gastric cancer cells in vitro by the activation of the Wnt-Ca^{2+} pathway (Kurayoshi *et al.*, 2006). In the other report, human gastric cancer cells with and without expressing WNT5A siRNA were injected in spleens of nude mice and the resulting liver metastases were counted. The authors could show that WNT5A increased the number of liver metastases due to its positive effect on the expression Laminin γ2, a component of the basement membrane, essential for cell migration and adhesion. In vitro, they showed that this effect is dependent on the Wnt-Ca^{2+} and Wnt-PCP pathway (Yamamoto *et al.*, 2009).

- Neuroblastoma

In human neuroblastoma cell cultures, WNT5A was shown to induce differentiation and repress invasion through the Wnt-Ca^{2+} pathway. It therefore acts as a tumor suppressive protein (Blanc *et al.*, 2005).

- Cholangiocarcinoma

WNT5A inhibits the proliferation of human cholangiocarcinoma cell cultures through the activation of the non-canonical Wnt-PCP pathway (DeMorrow *et al.*, 2008).

- Prostate cancer

In human prostate cancer cell lines, WNT5A was shown to induce the expression of BMPs in a Wnt-PCP pathway-dependent manner, leading to osteoblast differentiation and excessive bone production, thus contributing to the pathogenesis of this disease (Dai *et al.*, 2008). On the other hand, WNT5A inhibits androgen receptor (AR) transcriptional activity in human prostate cancer cell lines, supporting a tumor-suppressive function for WNT5A in the pathogenesis of this disease (Kawano *et al.*, 2009).

- Lung cancer

Tobacco smoke condensate (TSC)-induced expression of WNT5A may contribute to the pathogenesis of lung cancer through the activation of the Wnt-PCP pathway (Hussain *et al.*, 2009).

- Osteosarcoma

A study in human osteosarcoma cell lines showed that WNT5A increased invasion and migration through the Wnt-PCP pathway-dependent upregulation of MMP13 and a concomitant inhibition of the canonical Wnt pathway (Enomoto *et al.*, 2009).

- Papillary thyroid cancer

Via an unknown signalling pathway, WNT5A was shown to promote proliferation and migration in human papillary thyroid cancer cell cultures (McCall *et al.*, 2007).

- Pancreatic cancer

The two studies published in the context of pancreatic cancer lead to the conclusion that WNT5A promotes the formation of this cancer, but via an unknown pathway. The first study showed that TGFβ leads to an increased expression of WNT5A which in turn induces migration, invasion and proliferation and modulates expression of marker genes associated with epithelial-mesenchymal transition (EMT) (Ripka *et al.*, 2007). In the second study, the authors provide evidence that WNT5A induces the proliferation and migration of human pancreatic cancer cell cultures (Schwartz *et al.*, 2009).

- Glioblastoma

WNT5A promotes proliferation of human glioblastoma-derived tumor cells in vitro and induces infiltrating tumors when injected in nude mice (Yu *et al.*, 2007).

- Non-small-cell lung cancer

WNT5A induces proliferation in human cell cultures of non-small-cell lung cancer (Huang *et al.*, 2009).

- Hepatocellular carcinoma

In a study using well-differentiated and poorly-differentiated human hepatocellular carcinoma cell lines, WNT5A was shown to induce the poor-differentiated subtype by inhibiting the canonical Wnt pathway. The authors propose that Wnt pathways have complementary roles in the pathogenesis of hepatocellular carcinomas: a deregulated canonical Wnt pathway contributes to tumor initiation, whereas a non-canonical Wnt pathway induced by WNT5A is essential for tumor progression (Yuzugullu *et al.*, 2009).

Tumor, Role of WNT5A	*Pathway*	*Effect*	*Paper*
Breast cancer: ***Tumor promoter*** ***Tumor suppressor***	unknown	**Transformation** through **inhibition** of WNT5A **expression** (siRNA)	Olson and Gibo, 1998
	Canonical Wnt	**Promoting proliferation** through transactivation of EGFR	Civenni *et al.*, 2003
	Wnt-Ca2+	**Suppressing migration/invasion**	Dejmek *et al.*, 2006
	Wnt-PCP	WNT5A expressed by tumor-associated macrophages → **promoting invasion**	Pukrop *et al.*, 2006
	unknown	**Suppressing migration/invasion** in vitro, number of **metastases** (mouse model)	Safholm *et al.*, 2008
	unknown	**Promoting differentiation** through inhibition of the canonical Wnt-pathway	Roarty *et al.*, 2009
	PKA	**Suppressing migration**	Hansen *et al.*, 2009
	unknown	**Promoting ERα-expression** → responsive to Tamoxifen	Ford *et al.*, 2009a
	unknown	**Suppressing number of metastases** (mouse model)	Ford *et al.*, 2009b
	unknown	**Suppressing invasion** through increasing intercellular adhesion	Medrek *et al.*, 2009
Cutaneous melanoma: ***Tumor promoter***	Wnt-Ca2+	**Promoting migration/invasion**	Weeraratna *et al.*, 2002
	Wnt-Ca2+ Wnt-PCP	**Promoting migration**	Witze *et al.*, 2008
	Wnt-Ca2+ JAK-STAT	**Suppressing** expression **tumor-associated antigens** MITF, MART-1	Dissanayake *et al.*, 2008
	Wnt-Ca2+ Wnt-PCP	**Promoting migration**	O'Connell *et al.*, 2009a
	unknown	WNT5A expression modulated by heparan sulfate proteoglycans → **promoting invasion**	O'Connell *et al.*, 2009b
	Wnt-PCP	**Promoting metastasis** in vitro and in vivo	O'Connell *et al.*, 2009c
	unknown	**Promoting proliferation/migration**	Schwartz *et al.*, 2009
Hematopoietic neoplasia: ***Tumor suppressor***	Wnt-Ca2+	**Suppressing proliferation** through inhibition of the canonical Wnt-pathway	Liang *et al.*, 2003
	unknown	**Promoter hypermethylated** → increased canonical Wnt-pathway activity	Roman-Gomez *et al.*, 2007
	unknown	**Promoter hypermethylated**	Ying *et al.*, 2007
Gastric cancer: ***Tumor promoter***	Wnt-Ca2+	**Promoting migration**	Kurayoshi *et al.*, 2006
	Wnt-Ca2+ Wnt-PCP	**Promoting number of metastases** (mouse model)	Yamamoto *et al.*, 2009

Neuroblastoma: ***Tumor suppressor***	Wnt-Ca2+	**Promoting differentiation, suppressing invasion**	Blanc *et al.*, 2005
Cholangiocarcinoma: ***Tumor suppressor***	Wnt-PCP	**Suppressing proliferation**	DeMorrow *et al.*, 2008
Prostate cancer: ***Tumor promoter*** ***Tumor suppressor***	Wnt-PCP	**Promoting excessive bone production**	Dai *et al.*, 2008
	unknown	**Suppressing AR-dependent transcription**	Kawano *et al.*, 2009
Lung cancer: ***Tumor promoter***	Wnt-PCP	Wnt5A expression **induced by tobacco smoke consensate (TSC)**	Hussain *et al.*, 2009
Osteosarcoma: ***Tumor promoter***	Wnt-PCP	**Promoting migration/invasion** through inhibition of the canonical Wnt-pathway	Enomoto *et al.*, 2009
Papillary thyroid cancer: ***Tumor promoter***	unknown	**Promoting proliferation/migration**	McCall *et al.*, 2007
Pancreatic cancer: ***Tumor promoter***	unknown	**Promoting proliferation/migration/ invasion/EMT**	Ripka *et al.*, 2007
	unknown	**Promoting proliferation/migration**	Schwartz *et al.*, 2009
Glioblastoma: ***Tumor promoter***	unknown	**Promoting proliferation** in vitro, **invasion** in mouse model	Yu *et al.*, 2007
Non-small-cell lung cancer: ***Tumor promoter***	unknown	**Promoting proliferation**	Huang *et al.*, 2009
Hepatocellular carcinoma: ***Tumor promoter***	unknown	**Promotes poor differentiation** subtype through inhibition of the canonical Wnt-pathway	Yuzugullu *et al.*, 2009

Figure 37: The role of WNT5A in the pathogenesis of cancer based on functional studies in cell cultures and animal models

1.2.2. Expression studies in human tumor tissues

Figure 38 lists the WNT5A expression studies performed in various tumor tissues to correlate its expression with clinical data.

- Studies that confirmed the results obtained from cell culture experiments and animal models

Expression data from breast cancer tissues favour a role for WNT5A as a tumor suppressor in this disease (Jonsson *et al.*, 2002; Leris *et al.*, 2005). This confirms most of the data obtained from functional studies.

Studies done using tissues of cutaneous melanoma also verify the in vitro findings that WNT5A acts as a tumor promoter in cutaneous melanoma (Da Forno *et al.*, 2008; Dissanayake *et al.*, 2008; Weeraratna *et al.*, 2002).

Expression and promoter-methylation studies performed in tissues of hematopoietic neoplasias reflect the results obtained in vitro using cell cultures (Liang *et al.*, 2003; Roman-Gomez *et al.*, 2007; Ying *et al.*, 2007).

For gastric cancer, its role as a tumor promoter in cell cultures and mouse model could be confirmed performing expression studies in tumor tissues (Kurayoshi *et al.*, 2006; Yamamoto *et al.*, 2009).

A positive correlation between the results of in vitro studies and expression analyses in tumor tissues could also be obtained for neuroblastoma, pointing towards a tumor suppressive role of WNT5A in the pathogenesis of this disease (Blanc *et al.*, 2005).

In vivo analyses of prostate cancer revealed a hypomethylation of the WNT5A promoter leading to its increased expression as compared to reference cells, thus indicating a tumor promoting function of WNT5A (Wang *et al.*, 2007). This outcome verifies the result of a study performed in vitro (Dai *et al.*, 2008; Kawano *et al.*, 2009).

The proposed role of WNT5A as a tumor promoter in pancreatic cancer cell culture experiments was confirmed by expression studies in tumor tissues (Crnogorac-Jurcevic *et al.*, 2001; Ripka *et al.*, 2007).

Also glioblastoma cell culture and mouse model data could be confirmed in vivo, whereupon WNT5A acts as a tumor promoter in this disease (Yu *et al.*, 2007).

- *Studies that did not confirm the results obtained from cell culture experiments and animal models*

Whereas the in vitro study using cell cultures of non-small-cell lung cancers favoured a tumor-promoting role for WNT5A in the pathogenesis of this type of cancer, the interpretation of in vivo data are not conclusive (Campioni *et al.*, 2008; Huang *et al.*, 2005).

A contradictory result to the one obtained in vitro (tumor promoter) yielded the expression study in hepatocellular carcinoma, favouring a tumor suppressive role for WNT5A (Liu *et al.*, 2008a).

- *Studies performed in tumors that have not been analyzed in vitro or in animal models so far*

Studies in ovarian cancer (Badiglian Filho *et al.*, 2009) and head and neck squamous cell carcinoma (Diaz Prado *et al.*, 2009), both proposing a tumor promoting role for WNT5A, can not be compared with functional experiments.

Tumor, Role of WNT5A	*Expression*	*Paper*
Breast cancer: ***Tumor suppressor***	Loss of expression in invasive ductal carcinomas is associated with higher histological grade, absence of estrogen- and progesterone-receptors and recurrent disease	Jonsson *et al.,* 2002
	Expression in malignant tumors < normal tissues. Lower expression correlates with progressive disease. ER-neg. cases: lower expression correlates with worse clinical outcome	Leris *et al.,* 2005
Cutaneous melanoma: ***Tumor promoter***	Expression at sites of active invasion > other sites	Weeraratna *et al.,* 2002
	Correlation with progression, risk factor for reduced metastasis-free and overall survival	Da Forno *et al.,* 2008
	Expression correlates with metastasis and inversely correlates with expression of tumor-associated antigens MART-1, GP100	Dissanayake *et al.,* 2008
Hematopoietic neoplasia: ***Tumor suppressor***	80% of acute lymphoblastic leukemia (ALL): lack of expression ; 100% of acute myeloid leukemia (AML): reduced or lack of expression	Liang *et al.,* 2003
	ALL: promoter hyermethylation in 43% of tissues. Hypermethylation correlates with upregulation of cyclin D1. Disease-free survival in hypermethylated patients reduced	Roman-Gomez *et al.,* 2007
	Burkitt lymphoma (BL), NK/T-cell lymphoma (NL), other non-Hodgkin‘s lymphoma (NHL): Promoter methylated in 50% of BL, 73% of NL, 31% NHL.	Ying *et al.,* 2007
Gastric cancer: ***Tumor promoter***	Correlation with advanced stages and poor prognosis	Kurayoshi *et al.,* 2006
	Correlation with advanced T- and N-classification, tumor stage and Laminin γ2 expression	Yamamoto *et al.,* 2009
Neuroblastoma: ***Tumor suppressor***	Reduced expression is correlated with a highly aggressive behavior	Blanc *et al.,* 2005

Prostate cancer: ***Tumor promoter***	Promoter is hypomethylated → expression > normal cells	Wang *et al.,* 2007
Pancreatic cancer: ***Tumor promoter***	Adenocarcinoma > normal pancreas	Crnogorac-Jurcevic *et al.*, 2001
	Expression early during cancerogenesis in pancreatic intraepithelial neoplasias and in invasive pancreatic adenocarcinomas > normal pancreatic tissues	Ripka *et al.*, 2007
Glioblastoma: ***Tumor promoter***	Expression > normal brain tissue	Yu *et al.,* 2007
Non-small-cell lung cancer: ***Tumor promoter*** ***Tumor suppressor***	Correlation with Ki-67 proliferation index and a significant prognostic factor	Huang *et al.,* 2005
	Expression downregulated from stage 1 to stage 2	Campioni *et al.,* 2008
Hepatocellular carcinoma: ***Tumor suppressor***	Reduced or loss of expression in 81% of cases analyzed. Low expression associated with higher tumor stage, low membranous expression of E-cadherin and β-catenin and high Ki-67 staining	Liu *et al.*, 2008b
Ovarian cancer: ***Tumor promoter***	Expression epithelial ovarian cancer > benign epithelial neoplasia, normal ovaries. Predictor of poor prognosis	Badiglian Filho *et al.,* 2009
Head and neck squamous cell carcinoma: ***Tumor promoter***	Expression in central and peritumoral tissues > healthy oral mucosa	Diaz Prado *et al.,* 2009

Figure 38: The role of WNT5A in the pathogenesis of cancer based on expression studies in human tumor tissues and their correlation with clinical data

2. Introduction

2.1. WNT5A is highly overexpressed in aggressive fibromatoses

The microarray gene expression study comparing pooled aggressive fibromatosis tumor tissues with pooled superficial fibromatosis and reference fibrous tissues revealed that WNT5A is highly overexpressed in aggressive fibromatoses (*Chapter I, Section 4.2.*). Real-time RT-PCR (*Chapter I, Section 4.2.*) and additional microarray experiments (*Chapter II, Section 4.3*) on individual tumor tissue samples confirmed an upregulation of WNT5A in aggressive fibromatoses.

2.2. WNT5A has been implicated in the pathogenesis of many types of cancer

Numerous studies published in the literature brought WNT5A into context with the pathogenesis of different types of cancer. Experiments based on functional studies in cell cultures and animal models are reviewed in *Section 1.2.1.*, whereas reports linking the expression of WNT5A with clinical data are summarized in *Section 1.2.2.* of this chapter.

Aggressive fibromatoses and colon carcinoma are both characterized by β-catenin and APC mutations in most of the cases (*Chapter I, Sections 1.1.3. and 2.2.1.*). Studies have been done in colon carcinoma to elucidate the functional role of WNT5A. They all suggest that WNT5A exerts a tumor-suppressive role in the pathogenesis of this disease. An initial report described that WNT5A-expression was missing in several colon cancer cell lines analyzed (Smith *et al.*, 1999). This finding was confirmed by a later study showing that the WNT5A promoter is hypermethylated in the cancer cell lines HCT116, HT29, SW620, Caco2 and LoVo (Ying *et al.*, 2008). Two studies provided evidence that WNT5A achieves its tumor suppressive function in colon cancer through the inhibition of the canonical Wnt pathway activity: in the first, it was shown that WNT5A leads to an increased degradation of β-catenin in a ROR2- and Siah2-dependent manner in the colon cancer cell line HT-29 (MacLeod *et al.*, 2007). The second study reports about an inhibitory effect of WNT5A on the canonical Wnt-signalling pathway in the cell line HCT116, resulting in a suppressed tumor cell colony formation of these cells (Ying *et al.*, 2008). Finally, by an unknown mechanism, WNT5A suppressed migration of the colon carcinoma cell line SW480 (Dejmek *et al.*, 2005). Expression and promoter methylation studies performed in tissues of colon carcinoma reflect the results obtained in vitro using cell cultures: the WNT5A promoter is often hypermethylated, leading to a reduced expression of WNT5A in these tumors as compared to normal epithelia (Dejmek *et al.*, 2005; Smith *et al.*, 1999; Ying *et al.*, 2008).

2.3. The signalling pathways activated by WNT5A

WNT5A is known to activate both canonical and non-canonical Wnt pathways. The canonical Wnt pathway has comprehensively been introduced in *Chapter I, Section 1.1.,* whereas the non-canonical pathways Wnt-Ca^{2+}, Wnt-PCP, PKA and STAT, including their crosstalks with the canonical Wnt pathway, have been described in *Section 1.1.* of this chapter. *Figure 39* summarizes those signalling pathways.

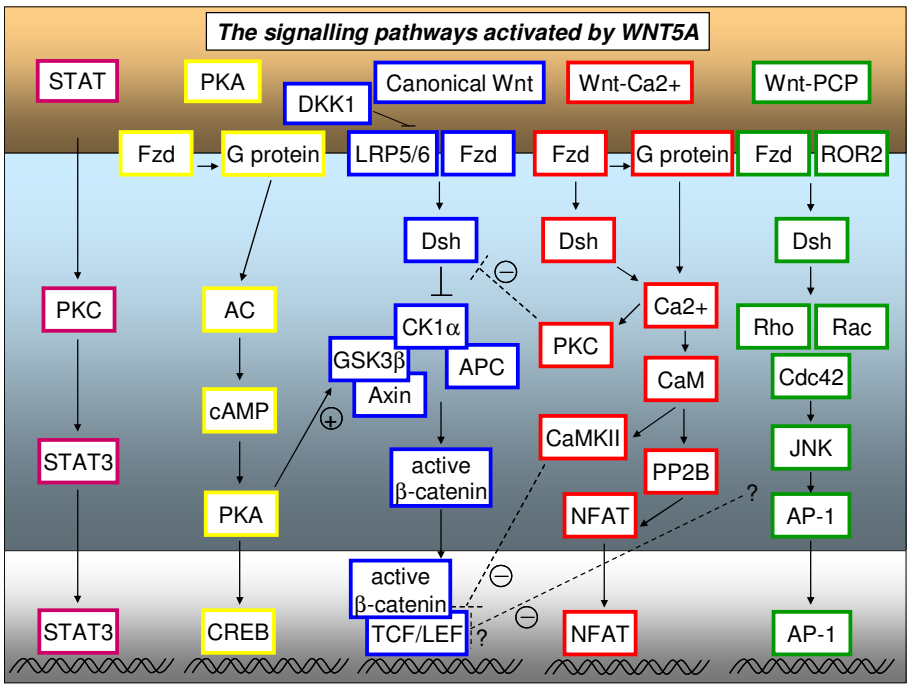

Figure 39: The signalling pathways activated by WNT5A. A plus-sign in a circle indicates that the non-canonical pathway is known to exert a stimulating effect on the activity of the canonical pathway, whereas the inverse is true for a minus-sign.

2.4. Widely used methods to measure the activities of WNT5A-stimulated signalling pathways

Some methods often described in the literature to measure the activity of WNT5A-stimulated signalling pathways are summarized in *Figure 40*.

The canonical Wnt pathway was analyzed by measuring the accumulation of the active, N-terminally dephosphorylated form of β-catenin in the cytoplasm using Western blot analysis on cytoplasmic protein extracts, since the process of β-catenin phosphorylation and degradation and its inhibition by the canonical Wnt pathway occurs in this cell compartment. Additionally, a TCF reporter-gene assay (Luciferase assay) was often used to measure the activity of the TCF-dependent transcription, known to be induced by an activated canonical Wnt pathway.

The activities of the non-canonical Wnt-Ca^{2+}, Wnt-PCP, and STAT pathways can be monitored using transcription factor (TF)-activity assays for NFAT (Wnt-Ca^{2+}), AP-1 (Wnt-PCP), and STAT3 (STAT), that are all based on nuclear protein extracts.

The activity of the PKA pathway was described to be measured on Western blots using nuclear protein extracts and an antibody that is specific for the active, serine 133-phosphorylated form of CREB.

In addition, WNT5A-induced crosstalks between the non-canonical and the canonical pathways can be analyzed by a specific inhibition of a central component of a non-canonical pathway, followed by WNT5A-treatment and the measurement of the canonical Wnt pathway activity.

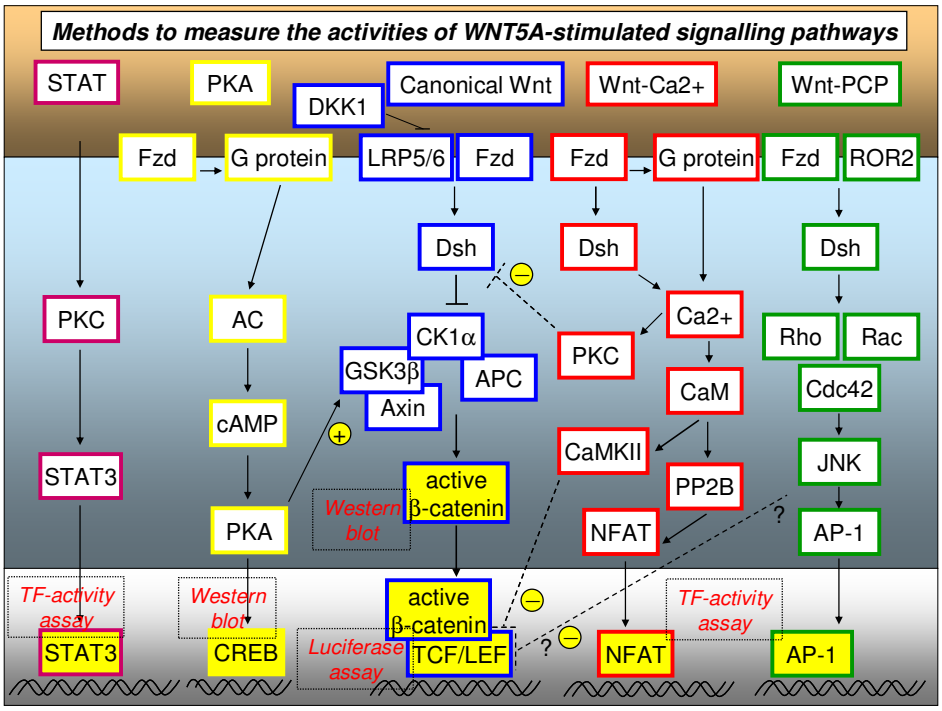

Figure 40: Often used methods to measure the activities of WNT5A-stimulated signalling pathways. A plus-sign in a circle indicates that the non-canonical pathway is known to exert a stimulating effect on the activity of the canonical pathway, whereas the inverse is true for a minus-sign.

2.5. Aim of the study

Our microarray gene expression analysis on pooled RNA samples revealed that WNT5A is highly overexpressed in aggressive fibromatosis as compared to superficial fibromatosis and reference fibrous tissue (*Chapter I, Section 4.2.*). This finding could be verified on individual tumor tissue samples using real-time RT-PCR (*Chapter I, Section 4.2.*) and additional microarray experiments (*Chapter II, Section 4.3*).

Therefore, the aim of the experiments described in this chapter was to analyze the impact of WNT5A on the activity of canonical- and non-canonical Wnt pathways, as well as on proliferation and invasive behaviour in the primary aggressive fibromatosis tumor cell culture Aggr6 in comparison to cultures of normal fibroblasts. In addition, a gene expression analysis using Agilent microarrays should bring light into the differential gene expression induced by WNT5A in those cell cultures.

3. Materials and methods

3.1. Protein extractions

All protein extracts were quantified following the protocol of the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL). The absorbance of the colorimetric reaction within the wells was measured on an ELX800NB spectrophotometer (BioTec, Winooski, VT) at 570 nm. Prior to quantification, the whole cell protein extracts were heated at 95°C for 5 minutes, followed by centrifugation at 21'000 g for 5 min. The supernatants were transferred into new tubes, whereas the pellets were discarded.

3.1.1. Whole cell protein extracts

After washing in Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA), cells were scrabbed from the flask using 1 ml 2x sample buffer (100mM Trizma acid (TrisHCl) pH 6.8, 4% SDS, 10% Glycerol) per 75 cm^2 flask and stored at -20°C. Those extracts were used for the analysis of the subcellular protein fractionation process performance (*Section 4.1.*).

3.1.2. Subcellular phospho-protein fractions

Subcellular phospho-protein fractions were extracted according to the instruction manual of the Nuclear Extract Kit (Active Motif, Carlsbad, CA). An overview of the experimental steps is given in *Figure 41.* The cells were opened in a hypotonic buffer containing a weak detergent (1). After a centrifugation step (2), the supernatant contained the soluble cytoplasmic protein extract, whereas in the pellet, the intact nuclei and fragments of the cytoplasm membrane were concentrated. The nuclei were then lysed by a strong detergent in lysis buffer (3) and a final centrifugation step (4) differentiated the soluble nuclear extract from the pellet containing cytoplasm- and nuclear membrane fragments.

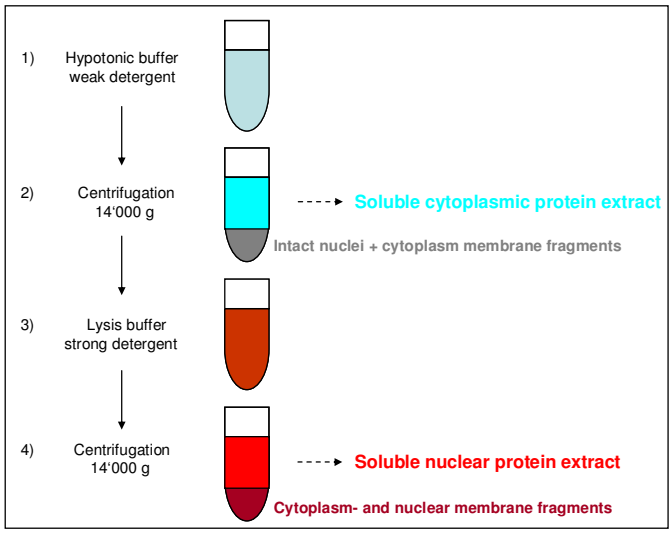

Figure 41: Principle working steps needed to perform subcellular phospho-protein fractionation according to the protocol of the Nuclear Extract Kit (Active Motif)

The soluble nuclear and cytoplasmic phospho-protein fractions represent the basis for subsequent Western blot experiments and transcription factor activity assays depicted in *Section 4* of this chapter. They were stored at -80°C. For the analysis of the subcellular protein fractionation process performance (*Section 4.1.*), membranous protein fractions, usually generated as pellets to be discarded during the process of protein fractionation, were used as well. They were resolved in 2x sample buffer (100mM Trizma acid (TrisHCl) pH 6.8, 4% SDS, 10% Glycerol) and stored at -20°C.

3.2. SDS-PAGE and Western blot

Proteins were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on Precast Gels 4-20% Tris-HCl (BIORAD, Hercules, CA) using a BIORAD apparatus. Per lane, 5 µg of protein in a volume of 15 µl was loaded, including 3 µl 5x sample buffer containing bromophenol blue plus β-mercaptoethanol (250mM Trizma acid (TrisHCl) pH 6.8, 10% SDS, 25% Glycerol, 0.25% bromophenol blue, 10% β-mercaptoethanol). Accordingly, the samples were diluted in 1x sample buffer containing bromophenol blue (50mM Trizma acid (TrisHCl) pH 6.8, 2% SDS, 5% Glycerol, 0.05% bromophenol blue). Prior to loading, the samples were heated at 95°C for 5 minutes. A mixture of the SeeBlue Plus2 Pre-Stained Standard and the MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA) was always loaded to evaluate the molecular size of the resolved proteins. The SDS-PAGE was run at 200 V and 0.05 A using the Pac 200 power supply (BIORAD, Hercules, CA). The proteins were transferred onto Whatman Protran Nitrocellulose Transfer Membranes (GE Healthcare, Waukesha, WI) at 60 V and 0.4 A for 45 minutes. Membranes were blocked for 1 hour at room temperature (RT) by gentle shaking in 5% non-fat milk powder containing blocking buffer 1xTBS, 0.1% Tween (3 g TrisHCl pH 7.4, 8 g NaCl, 0.2 g KCl, 1 ml Tween 20 per liter H_2O). Membranes were incubated in primary antibody solution overnight at 4°C by gentle shaking. The next day, membranes were washed 3 times 30 min in 1xTBS, 0.1% Tween, before incubating them in secondary antibody solution for 1 hour at RT under gentle shaking. Primary and secondary (horseradish peroxidase-labelled) antibodies are cited in *Table 3*. Membranes were washed as above and incubated in SuperSignal West Dura Extended Duration Substrate Solutions (Pierce, Rockford, IL) for detecting the active-β-catenin and active-CREB antibodies and in Amersham ECL Western Blotting Analysis System Detection Reagents (GE Healthcare, Waukesha, WI) for the detection of the other antibodies. After exposure of the membranes to Hyperfilm ECL high performance chemiluminescence films (GE Healthcare, Waukesha, WI), the films were developed on a FILM FPM 800A Medical Film Processor (FUJI, Tokyo, Japan). They were scanned on a Perfection 4870 Photo Scanner (Epson, Long Beach, CA) and the bands of interest quantified on the Quantitiy One software (BIORAD, Hercules, CA).

Antibodies	Dilution
1) active (S37,T41-dephospho)-**β-catenin** (mouse, monoclonal, A)	1:500 TBS/T, 5% MP
2nd horse anti-mouse IgG (B)	1:5'000 TBS/T, 1% MP
2) active (S133-phospho)-**CREB** (rabbit, monoclonal, B)	1:1'000 TBS/T, 5% BSA
2nd goat anti-rabbit IgG (A)	1:2'000 TBS/T, 5% MP
3) β-tubulin (mouse, monoclonal, C)	1:1'000 TBS/T, 0.5% MP
2nd horse anti-mouse IgG (B)	1:5'000 TBS/T, 0.5% MP
4) HDAC1 (mouse, monoclonal, D)	1:100 TBS/T, 0.5% MP
2nd horse anti-mouse IgG (B)	1:5'000 TBS/T, 0.5% MP
5) Lamin A/C (mouse, monoclonal, E)	1:500 TBS/T, 0.5% MP
2nd horse anti-mouse IgG (B)	1:5'000 TBS/T, 0.5% MP
6) GLUT1 (rabbit, polyclonal, A)	1:1'000 TBS/T, 5% MP
2nd goat anti-rabbit IgG (A)	1:3'000 TBS/T, 0.5% MP
7) WNT5A (goat, polyclonal, F)	1:1'000 TBS/T, 5% MP
2nd donkey-anti-goat IgG (B)	1:5'000 TBS/T, 5% MP

Table 3: Primary and secondary antibody solutions used for Western blots. Abbreviations: TBS/T = 1xTBS, 0.1% Tween (see text for details); MP = non-fat milk powder; BSA = bovine serum albumin. Companies providing the antibodies: A = Millipore, Billerica, MA; B = Cell Signalling, Danvers, MA; C = Sigma-Aldrich, Saint Louis, MI; D = Abcam, Cambridge, UK; E = BD Transduction Laboratories, Franklin Lakes, NJ; F = R&D Systems, Minneapolis, USA.

A detailed description of the kinetics and treatment concentrations applied in the Western blot experiments depicted in *Section 4* can be obtained in *Section 3.4., Table 4*.

3.3. TCF-reporter gene assay (Luciferase assay)

Cells were transfected using the Amaxa Basic Nucleofector Kit for Primary Mammalian Fibroblasts on the Amaxa Nucleofector electroporation system (Lonza, Basel, Switzerland). The constructs needed to measure the activity of the canonical signalling pathway in transfected cells were kindly provided by Dr. van der Flier (Aglaia Biomedical Ventures, Utrecht Area, Netherlands): the 'TOP'-construct containing unmutated TCF responsive elements and minimal TATA box upstream of the firefly luciferase reporter gene, and the 'FOP'-construct being mutated in the TCF responsive elements and therefore unable to bind TCF and respond to canonical Wnt signalling activity. To normalize differences from transfection to transfection concerning transfection efficiency and numbers of cells used, the pRL-SV40-renilla luciferase reporter vector 'Renilla' (Promega, Madison, WI) was always cotransfected with TOP and FOP, respectively.

The day before transfection, cells were splitted to reach 80-90% confluency at transfection. That day, $1x10^6$ cells were resuspended in 100 µl of Amaxa Supplemented Nucleofector Solution (Lonza, Basel, Switzerland). To the suspension, 1 µg of TOP or FOP was added, in addition to 0.5 µg of Renilla. The cells were electroporated on the Amaxa Nucleofector using protocol A-34. After transfection, the cells were transferred back into cell culture flasks to recover for at least 8 hours. Firefly- and Renilla-luciferase activity in the transfected cells were measured according to the technical manual of the Dual-Luciferase Reporter Assay System on the Glomax apparatus (Promega, Madison, WI). The activity of the canonical Wnt signalling pathway was defined as follows: Ratio TOP/Renilla (Transfection 1) divided by Ratio FOP/Renilla (Transfection 2). A resulting value of 1 indicates that the canonical Wnt signalling pathway is not active in the corresponding cells, whereas values greater than 1 reflect activated canonical Wnt signalling.

A comprehensive description of the kinetics and treatment concentrations applied in the Luciferase assays depicted in *Section 4* can be obtained in the following *Section 3.4.* (*Table 4*).

The Δ45-β-catenin expression plasmid used to activate the canonical Wnt signalling pathway in cell cultures of normal fibroblasts NHDF and Aggr2 was kindly provided by Prof. Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD). In those experiments, the construct was cotransfected with TOP and FOP, respectively. The cells were treated 16 hours and 40 hours after transfection, respectively, with 150ng/ml recombinant WNT5A (R&D Systems, Minneapolis, USA) for 8 hours, before Luciferase activity measurements were performed.

3.4. Detailed kinetics and treatment concentrations in experiments using Western blots and Luciferase assays

Experiment		Treatment	Hours after transfection/last splitting		
			Start treatment	Luciferase measurement/ Protein extraction	Duration treatment (h)
control	Luciferase	no		23	
	Western β-catenin	no		32	
WNT5A	Luciferase	150ng/ml	15	23	8
	Western β-catenin	150ng/ml	24	32	8
DKK1+WNT5A	Luciferase	150ng/ml + 150ng/ml	8 + 24	32	24 + 8
	Western β-catenin	150ng/ml + 150ng/ml	8 + 24	32	24 + 8
DKK1	Luciferase	150ng/ml	8	32	24
	Western β-catenin	150ng/ml	8	32	24
Antibody+WNT5A	Luciferase	2µg/ml + 150ng/ml	15	23	8
	Western β-catenin	2µg/ml + 150ng/ml	24	32	8
Antibody	Luciferase	2µg/ml	15	23	8
	Western β-catenin	2µg/ml	24	32	8
control	Luciferase	no		23	
	Western β-catenin	no		23	
WNT5A	Luciferase	150ng/ml	15	23	8
	Western β-catenin	150ng/ml	15	23	8
JNKI1+WNT5A	Luciferase	10µM + 150ng/ml	14.5 + 15	23	8.5 + 8
	Western β-catenin	10µM + 150ng/ml	14.5 + 15	23	8.5 + 8
JNKI1	Luciferase	10µM	14.5	23	8.5
	Western β-catenin	10µM	14.5	23	8.5
control	Western CREB	no		23	
Bt2cAMP	Western CREB	1mM	21	23	2
H-89+Bt2cAMP	Western CREB	10µM+1mM	20.5 + 21	23	2.5 + 2
WNT5A	Western CREB	150ng/ml	15	23	8
H-89+WNT5A	Western CREB	10µM + 150ng/ml	14.5 + 15	23	8.5 + 8
H-89	Western CREB	10µM	14.5	23	8.5
control	Luciferase	no		23	
	Western β-catenin	no		23	
Bt2cAMP	Luciferase	1mM	15	23	8
	Western β-catenin	1mM	21	23	2
H-89+Bt2cAMP	Luciferase	10µM+1mM	14.5 + 15	23	8.5 + 8
	Western β-catenin	10µM+1mM	20.5 + 21	23	2.5 + 2
WNT5A	Luciferase	150ng/ml	15	23	8
	Western β-catenin	150ng/ml	15	23	8
H-89+WNT5A	Luciferase	10µM + 150ng/ml	14.5 + 15	23	8.5 + 8
	Western β-catenin	10µM + 150ng/ml	14.5 + 15	23	8.5 + 8
H-89	Luciferase	10µM	14.5	23	8.5
	Western β-catenin	10µM	14.5	23	8.5

Table 4: Detailed kinetics and treatment concentrations in experiments using Western blots and Luciferase assays. Yellow: canonical Wnt pathway (*Section 4.2.1.*), green: crosstalk Wnt-PCP and canonical Wnt pathway (*Section 4.2.3.*), pink: PKA pathway (*Section 4.2.4.*), grey: crosstalk PKA and canonical Wnt pathway (*Section 4.2.4.*). Recombinant WNT5A, WNT5A antibody and recombinant DKK1 were obtained from R&D Systems, Minneapolis, USA. The JNK inhibitor 1 (JNKI1) was purchased from Axxora, LLC (San Diego, CA), whereas dihydrochloride hydrate (H-89) and dibutyryl cAMP sodium salt (Bt_2cAMP) were from Sigma-Aldrich, Saint Louis, MI.

3.5. Transcription factor activity assay

The following TransAM transcription factor activity assays from Active Motif (Carlsbad, CA) were used: NFATc1 (NFAT2), AP-1 for c-jun, JunB and JunD and STAT family for STAT1α and STAT3. They are based on 96-well-plates, where the consensus binding DNA-sequences for the relevant transcription factors are spotted on the bottom of the single wells. Only activated phosphorylated (AP-1, STAT) and dephosphorylated (NFAT), respectively, transcription factors are able to bind to these sequences. After the addition of a specific primary antibody and a horseradish peroxidase-conjugated secondary antibody, the absorbance of the colorimetric reaction within the wells was read on an ELX800NB spectrophotometer (BioTec, Winooski, VT) at 450 nm. The assays were performed according to the corresponding manufacturer's instructions: 2 µg of soluble nuclear phospho-protein extract was loaded per well. The specificity of the signals obtained were confirmed by the addition of wildtype and mutated consensus oligonucleotide sequences aligning (wildtype) or not aligning (mutated) to the spotted sequences at the bottom of the wells. Hybridized wildtype sequences hinder active transcription factors from binding to their spotted consensus sequences and therefore no signal should be obtained from those wells.

If applicable, the cells were treated for 10 min, 1 hour, 4 hours and 8 hours with 150 ng/ml recombinant WNT5A (R&D Systems, Minneapolis, USA), before soluble nuclear phospho-protein extracts were generated (*Section 3.1.2.*). For the activity measurement of c-jun in Aggr6, cells were additionally pre-treated for 30 minutes with 10µM JNK inhibitor 1 (JNKI1, Axxora LLC, San Diego, CA), before recombinant WNT5A was added.

3.6. BrdU-incorporation assay

The 5-Bromo-2'-deoxy-uridine (BrdU) Labelling and Detection Kit III (Roche, Basel, Switzerland) was used to measure proliferation. It is based on the principle that cells being engaged in the DNA-synthesis (S)-phase of the cell cycle incorporate 5-bromo-2-deoxy-uridine (BrdU) in place of thymidine into the newly synthesized DNA. These cells are then detected using an enzyme-conjugated antibody against BrdU. The higher the signal in a well, the more cells are involved in the S-phase of the cell cycle. It therefore reflects the proliferation rate of the cells within the corresponding well and inversely correlates with their doubling time.

The assay was performed according to the protocol provided by the company. 3'000 cells were seeded in 100 µl medium per well. After 48 hours of incubation, 10 µl of BrdU labelling solution was added to the cells for incorporation during 6 hours. Thereafter, the cells were fixed and treated with a peroxidase-conjugated antibody directed against BrdU. After the addition of peroxidase substrate, the absorbance of the colorimetric reaction within the wells was read on an ELX800NB spectrophotometer (BioTec, Winooski, VT) at 450 nm.

Before starting the experiment, all cells were cultivated for 16 hours in EGM2 basal medium and 10% FCS. Where necessary, cultured cells were pre-treated for the same period of time with DKK1 (150ng/ml), JNKI1 (10µM), H-89 (10µM), or a mixture of the three inhibitors. Cells were trypsinized and seeded into the wells for the BrdU-

assay. There, new medium was added, again containing the appropriate inhibitors. If applicable, cells were treated with recombinant WNT5A (600ng/ml) and an antibody against WNT5A (2 µg/ml).

3.7. Collagen type I cell invasion assay

The invasive capacity of the cell cultures Aggr6, Aggr2, and NHDF after stimulation with WNT5A was determined using the Boyden-chamber based Cultrex Collagen I Cell Invasion Assay (R&D Systems, Minneapolis, USA) (*Figure 40*). Such a collagen based assay was favoured over the extensively used matrigel based approach, since the matrigel represents a basement membrane model and is therefore convenient to study the invasive behaviour of epithelial cells, but not of fibroblasts. In vivo, fibroblasts are not forced to pass a basement membrane in order to be able to invade adjacent tissues.

Cells were serum starved for 24 hours before seeding into the inserts of the invasion chamber (4'500 cells in 50 µl serum- and supplement-free EGM2 basal medium). In the appropriate inserts, different concentrations of recombinant WNT5A was added (none, 300ng/ml or 600ng/ml). The bottom chambers were filled with 150 µl EGM2 basal medium supplemented with different concentrations of FCS (1% vs. 10% vs. 20%) and the same concentrations of WNT5A as applied in the upper inserts. 48 hours after seeding, invaded cells were labelled with calcein, a fluorescent compound that permeates cells, according to the manufacturer's protocol. The resulting signals were measured on a Tecan Genious apparatus (Tecan, Männedorf, Switzerland) at 485 nm excitation and 520 nm emission.

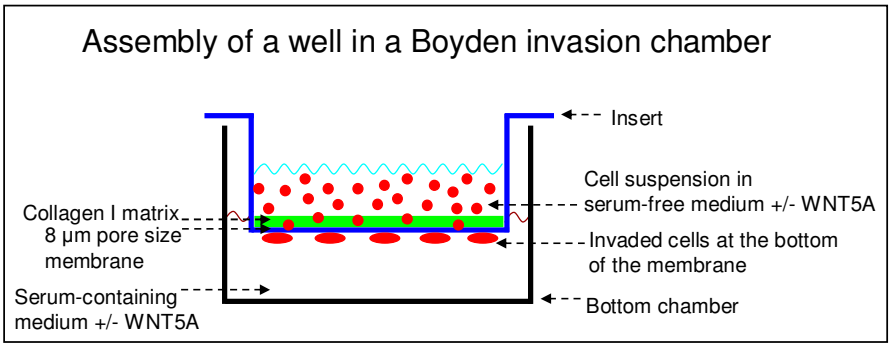

Figure 42: Assembly of a well in a Boyden invasion chamber assay used to analyze the invasive capacity of Aggr6, Aggr2, and NHDF cells with and without the addition of different concentrations of recombinant WNT5A in both the insert and the bottom chamber

3.8. Agilent 60mer-oligo microarrays

The day before the experiment, Aggr2, Aggr4, Aggr6 and NHDF cells were splitted into 25 cm^2 cell culture flasks at a density to reach ~80-90% confluency over night. Then, 600 ng/ml recombinant WNT5A (R&D Systems, Minneapolis, USA) was added and cells incubated for 8 hours. Control cells remained untreated. After washing in Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS), cells were trypsinized (1xTrypsin-EDTA, Invitrogen, Carlsbad, CA), centrifuged (160 g, 3 minutes) and the pelleted cells (~1x10^6) resuspended in 1 ml of TriReagent (Molecular Research Center, Inc., Cincinnati, OH) for subsequent total RNA extraction according to the protocol

supplied by the company. Resulting total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Agilent Technologies, Santa Clara, CA).

RNA quality control was performed as described in *Chapter I, Section 3.2.2.*

The samples were single color (Cy3)-labelled, hybridized, and the resulting data processed according to the descriptions in *Chapter I, Section 3.2.4.*

3.9. siRNA-experiments

Aggr6 cells were cotransfected using the Amaxa Basic Nucleofector Kit for Primary Mammalian Fibroblasts on the Amaxa Nucleofector electroporaton system (Lonza, Basel, Switzerland) with the TOP- or FOP-plasmid, the Renilla-construct (all introduced in *Section 3.3.*), and a mix of the following Dharmacon On-target plus siRNA SMARTpools (Thermo Scientific, Lafayette, CO) at different concentrations (0nM, 20nM, 200nM, 2µM), targeting Fzd1, Fzd2 and Fzd7 receptors:

Fzd1 (target sequences): GUUCUACCCUCUAGUGAAA, UUACGUACCUGGUGGA CAU, GAAGCCAACUCACAGUAUU, GCAAGACCCUCAACUCCUG

Fzd2 (target sequences): CCACGUACUUGGUAGACAU, GAACUGCGCUUCUUCC UGU, GGAGGAAGUUCUACACUCG, GCUACAAGUUUCUGGGCGA

Fzd7 (target sequences): UCAAGUACCUGAUGACCAU, GUUCGUCUACCUCUUC AUA, UGAUGUACUUUAAGGAGGA, AGGCAUAACUGUGACGAA

As a negative control, the On-target plus non-targeting pool of four non-specified, non-targeting siRNAs was transfected at the highest concentration (2µM).

16 hours, 40 hours, and 64 hours after transfection, the cells were treated with 150ng/ml recombinant WNT5A for 8 hours, before the luciferase activity measurement was performed.

4. Results

4.1. Analysis of the performance of subcellular protein fractionation

Since the analyses of the activities of the signalling pathways induced by WNT5A described in this chapter are partly dependent on subcellular protein fractions (Western blots, TF-activity assays), the following section describes the results of experiments dedicated to investigate the performance of the kit used to separate nuclear from cytoplasmic protein fractions. The subcellular protein fractions obtained through the different steps described in *Section 3.1.2.* were tested for their purity on Western blots by means of antibodies against the following marker proteins: β-tubulin (soluble cytoplasmic protein), histone deacetylase 1 (HDAC1, soluble nuclear protein), lamin A/C (largely a insoluble nucear membrane protein) and glucose transporter 1 (GLUT1, a membrane bound protein).

Figure 43 shows the results obtained in a typical separation experiment. HDAC1 was present in the whole cell extract (*lane 1*) and prominently in the soluble nuclear protein fraction (*lane 2*). It was also detectable in the fraction that includes the whole nuclei plus the cytoplasmic membrane fragments but, due to the dilution effect, in a lower concentration (*lane 4*). Nevertheless, the soluble cytoplasmic extract also contained low amounts of this soluble nuclear protein (*lane 3*). β-tubulin could only be found in the whole cell protein extract (*lane 1*) and in a high concentration in the soluble cytoplasmic fraction (*lane 3*). Finally, GLUT1 and lamin A/C are present only in the membrane-containing fractions (*lane 4* and *5*).

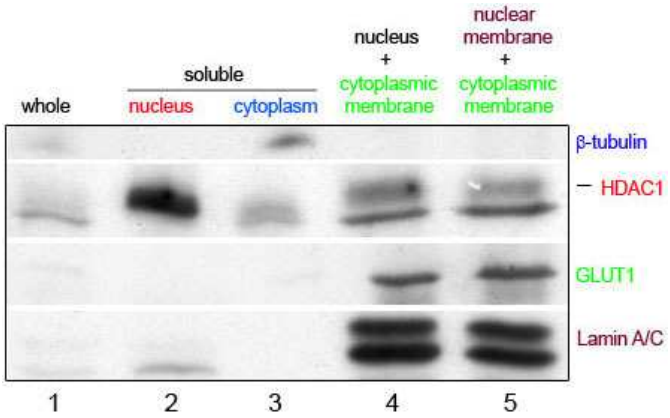

Figure 43: Distribution of the marker proteins β-tubulin (cytoplasm), HDAC1 (nucleus), GLUT1 (cytoplasmic membrane) and lamin A/C (nuclear membrane) in the different protein fractions

In summary, the soluble nuclear protein fraction is free from any contaminations derived from the other protein fractions. A substantial contamination of the soluble cytoplasmic protein fraction by soluble nuclear proteins can also be excluded.

4.2. Analysis of the impact of WNT5A on cellular signalling pathways in Aggr6 and cell cultures of normal fibroblasts

In the following section, the influence of WNT5A on the activation of the canonical and non-canonical Wnt signalling pathways as well as the induction of possible crosstalks between these pathways will be described. Aggressive fibromatosis tumor cells (Aggr6) and normal human fibroblasts are used as model system.

4.2.1. The effect of WNT5A stimulation on the canonical Wnt signalling pathway

- Baseline activity of canonical Wnt signalling in Aggr6 cells and normal fibroblasts

Using the TCF-reporter gene assay (Luciferase assay), the baseline activity of Wnt signalling in Aggr6 tumor cells and normal fibroblasts was determined (*Figure 44*). The canonical Wnt signalling pathway in cell cultures of normal dermal fibroblasts NHDF, HDF15 and HDF21 is almost inactive (1.0 on the scale of the y-axis indicates no measurable activity). On the other hand, the tumor-associated fibroblasts derived from aggressive fibromatoses Aggr2 and Aggr4 are characterized by a slight increase of canonical Wnt signalling activity as compared to the normal dermal fibroblasts. Since in Aggr2 and Aggr4, both alleles of the β-catenin gene are unmutated, the observed faint activation of this pathway may be derived from an endogenous paracrine/autocrine signalling induced by secreted canonical Wnt ligands. In contrast, a pronounced activity of the canonical Wnt signalling pathway can be measured in Agg6 tumor cells carrying a monoallelic β-catenin mutation at codon serine 45 (S45F). This is in agreement with the classical theory whereupon a monoallelic S45F mutation impedes phosphorylation at this residue by CK1α and subsequently also the phosphorylation at the other three residues by GSK3β, leading to the accumulation of dephosphorylated, active β-catenin. After translocation into the nucleus, it activates TCF-LEF-dependent transcription (*Chapter I, Sections 1.1.1. and 1.1.3.*).

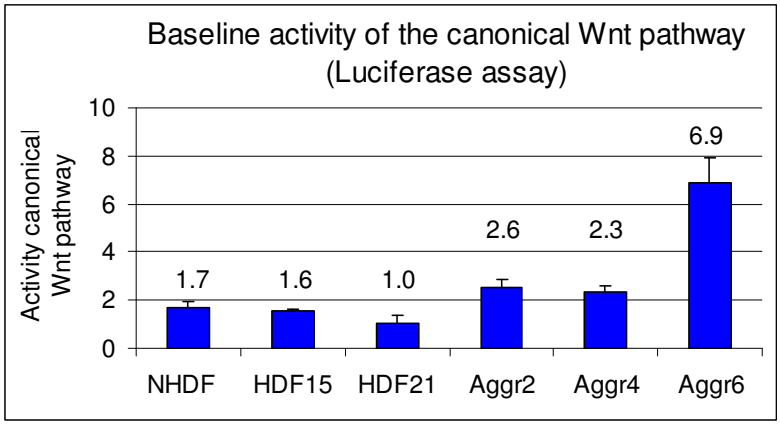

Figure 44: Baseline activity of the canonical Wnt signalling pathway in normal dermal fibroblasts NHDF, HDF15, HDF21, aggressive fibromatosis-associated normal fibroblasts Aggr2 and Aggr4 and aggressive fibromatosis tumor cells Aggr6 (Luciferase assay). The mean and standard deviation of three independent tests is demonstrated.

- The impact of endogenous Wnts on canonical Wnt signalling in Aggr6 cells

As demonstrated in the last section, Luciferase assay data revealed that Aggr6 tumor cells are characterized by a pronounced baseline activity of the canonical Wnt signalling pathway. The most obvious explanation for this finding is the monoallelic S45F-mutation in β-catenin carried by these cells. Another reason for this finding could be that secreted Wnt ligands lead to an increased canonical Wnt signalling activity. To test for this possibility, Aggr6 cells were treated with Dickkopf-1 (DKK1), a selective inhibitor of canonical Wnt signalling. DKK1 binds to the Frizzled (Fzd) coreceptor low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) and thus inhibits receptor activation through Wnt ligands. The effect of DKK1-treatment was measured both with Luciferase-assay (*Figure 45, L*) as well as with Western blot (*Figure 45, W*), using cytoplasmic protein extracts and an antibody against active, dephosphorylated β-catenin. DKK1-treatment of Aggr6 cells led to a reduction in the activity of the canonical Wnt pathway, demonstrated by both methods (*4*), as compared to untreated cells (*1*). This result reflects a measurable effect of endogenously produced Wnt ligands on the canonical Wnt signalling pathway activity in Aggr6 cells via wildtype β-catenin. Nevertheless, the level of activity is still clearly higher than in normal fibroblasts NHDF (*7*) and Aggr2 (*9*). This demonstrates a clear contribution of the mutated S45F-β-catenin to the increased canonical Wnt signalling activity in Aggr6 cells as well. In *Figure 46* this situation is graphically illustrated.

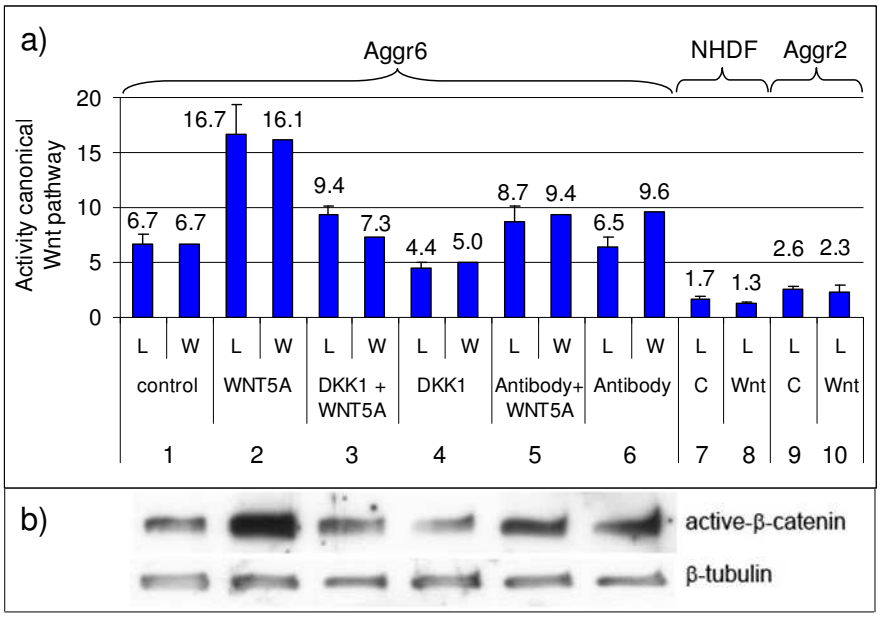

Figure 45: The impact of endogenous Wnts, WNT5A and recombinant WNT5A on the activity of the canonical Wnt pathway.

a) The activity of the canonical Wnt signalling was measured by Luciferase assays (L; mean and standard deviation of three independent experiments) and Western blots (W) in Aggr6 tumor cells (Aggr6) and normal human fibroblasts (NHDF, Aggr2). The different treatments are indicated. Control, C: untreated cells.

b) Western blot for active β-catenin, aligned to *Figure 45 a)*. The intensity of the bands was quantified and the resulting numbers integrated as columns (W) into *Figure 45 a)*. β-tubulin: loading control.

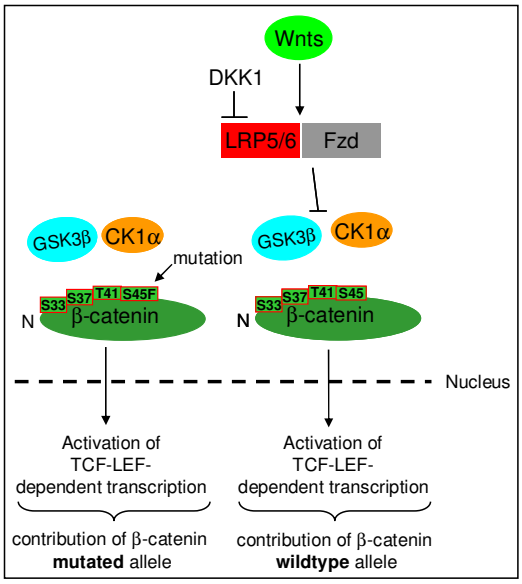

Figure 46: In Aggr6, both a monoallelic S45F-mutation in β-catenin and an endogenous Wnt signalling activity mediated by Wnt ligands contribute to the activation of TCF-LEF-dependent transcription

- The impact of endogenous WNT5A on canonical Wnt signalling in Aggr6 cells

To analyze whether endogenous WNT5A alone exerts a stimulating effect on the baseline canonical Wnt signalling activity in Aggr6, the cells were additionally treated with an antibody against WNT5A. *Figure 45* demonstrates that the WNT5A antibody did not affect the canonical Wnt signalling activity in Aggr6 cells (*6*), as compared to untreated cells (*1*). The increased activity determined using Western blot analysis (*6, W*) represents a technical artefact, since three independent Luciferase experiments (*6, L*) statistically prove that the antibody exerts no effect. Therefore, the endogenously produced WNT5A has no measurable effect on the canonical Wnt signalling pathway activity in Aggr6.

- The impact of recombinant WNT5A on canonical Wnt signalling in Aggr6 cells and in normal fibroblasts

Having shown that endogenous WNT5A does not exert any impact on canonical Wnt-signalling in Aggr6 cells, the question raises whether exogenous WNT5A is able to activate this pathway. Therefore, the cells were treated with recombinant WNT5A alone, or together with DKK1 to examine, whether WNT5A signals via the canonical Wnt pathway. The results depicted in *Figure 45* clearly demonstrate that recombinant WNT5A had indeed an inducing effect on the canonical Wnt signalling pathway (*2*), because its impact could be abrogated by the simultaneous addition of the canonical Wnt inhibitor DKK1 (*3*). The observed induction was specific, since the simultaneous addition of anti-WNT5A antibodies abolished the effect of WNT5A (*5*).

In contrast to the results obtained for Aggr6 cells, normal fibroblasts did not show any increase in the activity of the canonical Wnt signalling pathway after WNT5A stimulation (NHDF: *8*, Aggr2: *10*) as compared to untreated controls (NHDF: *7*, Aggr2: *9*).

4.2.2. The impact of recombinant WNT5A on the Wnt-Ca^{2+} pathway in Aggr6 cells

To determine, whether WNT5A signals via the Wnt-Ca^{2+} pathway, the activity of the downstream transcription factor NFAT2 was compared between WNT5A-treated and untreated Aggr6 cells. As *Figure 47* demonstrates, WNT5A does not influence the activity of this pathway in Aggr6 cells.

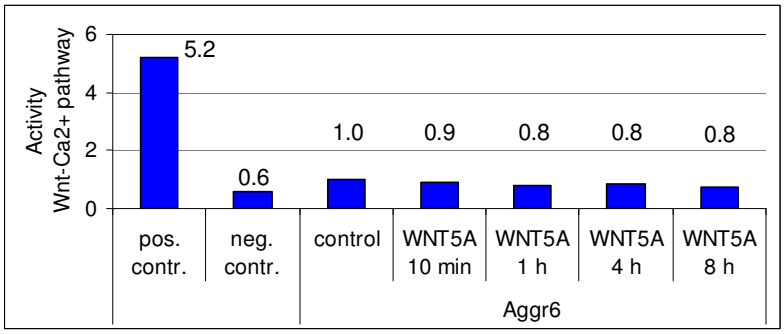

Figure 47: The impact of recombinant WNT5A on the activity of the Wnt-Ca^{2+} pathway in Aggr6, determined using a transcription factor-activity assay from Active Motif for NFAT2. Cells were treated with recombinant WNT5A for the indicated periods of time. As a positive control, a nuclear protein extract of phytohaemagglutinin (PHA)-treated Jurkat cells was used, whereas the value for the negative control is derived from a well containing no nuclear protein extract. Control: untreated cells.

4.2.3. The effect of WNT5A stimulation on the Wnt-PCP signalling pathway

- The impact of recombinant WNT5A on Wnt-PCP signalling in Aggr6 cells and in normal fibroblasts

The activities of the downstream transcription factors c-jun, junB and junD were compared between WNT5A-treated and untreated Aggr6 cells. WNT5A activated those transcription factors, showing a peak effect after ~ 1 hour of incubation (*Figure 48*, blue, brown and red curves). That WNT5A mediates this effect via the activation of the Wnt-PCP pathway was proven by pre-treating the cells with a specific inhibitor of JNK, JNK-inhibitor 1 (JNKI1), resulting in an abrogation of WNT5A's stimulating effect on c-jun (pink curve).

In contrast, recombinant WNT5A did not modulate the activity of the Wnt-PCP pathway in cell cultures of normal fibroblasts Aggr2 and NHDF, as proven by the analysis of the activity of c-jun in these cells (green and grey curves).

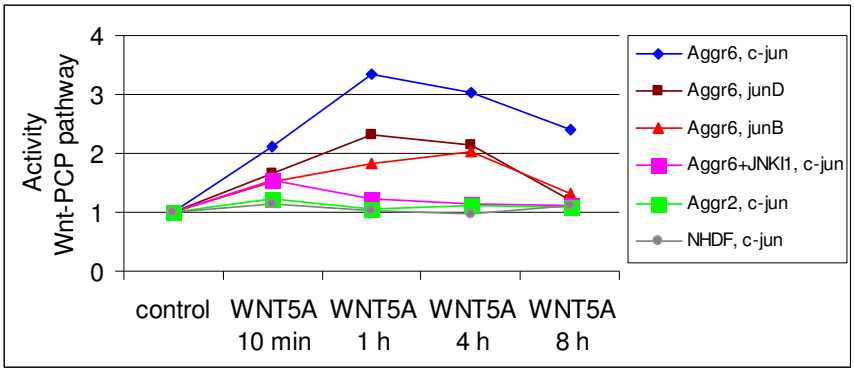

Figure 48: The impact of recombinant WNT5A on the activity of the Wnt-PCP pathway in Aggr6 and the cell cultures of normal fibroblasts Aggr2 and NHDF, determined using transcription factor-activity assays from Active Motif for c-jun, junD and junB. Cells were treated with recombinant WNT5A for the indicated periods of time. Control: untreated cells.

- *Analysis of the WNT5A-induced crosstalk between the Wnt-PCP and the canonical Wnt pathway in Aggr6 cells*

Having shown that recombinant WNT5A is able activate the canonical Wnt (*Section 4.2.1.*) and the non-canonical Wnt-PCP pathway in Aggr6 cells, it remains to be elucidated whether there is a WNT5A-induced crosstalk between these two pathways. To accomplish this purpose, Aggr6 cells were treated with recombinant WNT5A alone or with WNT5A after a pre-treatment with JNKI1 to selectively inhibit its impact on the Wnt-PCP pathway. Subsequently, the activity of the canonical Wnt pathway was measured both with Luciferase-assay (*Figure 49, L*) as well as with Western blot (*Figure 49, W*), using cytoplasmic protein extracts and an antibody against active, dephosphorylated β-catenin. Whereas the sole treatment with recombinant WNT5A increased the canonical Wnt pathway activity, demonstrated by both methods (*2*), as compared to untreated cells (*1*), the pre-treatment with JNKI1 did not alter WNT5A's effect (*3*). The somewhat reduced activity determined using Western blot analysis (*3, W*) represents a technical artefact, since three independent Luciferase experiments (*3, L*) statistically prove that the JNKI1 pre-treatment does not affect WNT5A's impact. This finding demonstrates that the selective inhibition of the Wnt-PCP pathway does not modulate the effect of WNT5A on the canonical Wnt pathway, indicating that a WNT5A-induced crosstalk between these two pathways in Aggr6 cells does not exist. In addition, the sole treatment of Aggr6 cells with JNKI1 (*4*) shows no measurable impact on canonical Wnt signalling in comparison to untreated cells (*1*), revealing that the baseline canonical Wnt signalling activity in Aggr6 cells is not influenced by any Wnt-PCP signalling activity.

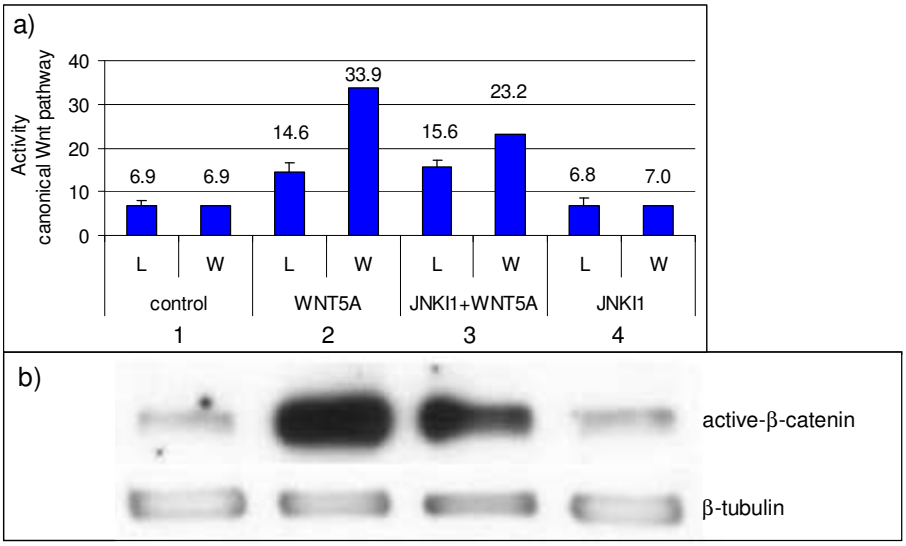

Figure 49: The analysis of the WNT5A-induced crosstalk between the Wnt-PCP and the canonical Wnt pathway in Aggr6 cells.

a) The activity of the canonical Wnt signalling was measured by Luciferase assays (L; mean and standard deviation of three independent experiments) and Western blot (W) in Aggr6 tumor cells. The different treatments are indicated. Control: untreated cells.

b) Western blot for active β-catenin, aligned to *Figure 49 a).* The intensity of the bands was quantified and the resulting numbers integrated as columns (W) into *Figure 49 a)*. β-tubulin: loading control.

4.2.4. The effect of WNT5A stimulation on the PKA signalling pathway

- The impact of recombinant WNT5A on PKA signalling in Aggr6 cells and in normal fibroblasts

Western blot analysis on nuclear protein extracts using an antibody specific for the active, serine 133-phosphorylated form of CREB was performed to investigate whether recombinant WNT5A activates the PKA pathway in Aggr6 cells and normal fibroblasts. This antibody crossreacts with the phosphorylated form of activating transcription factor 1 (ATF1), another phosphorylation target of PKA. Treatment of Aggr6 cells with recombinant WNT5A (*Figure 50, 4*) or dibutyryl cAMP sodium salt (Bt_2cAMP), a cell-permeable cAMP analog that activates PKA (*2*), led to an accumulation of both phosphorylated active CREB and ATF1, as compared to untreated cells (*1*). This demonstrates that WNT5A is able to phosphorylate and activate those two PKA target proteins. On the other hand, pre-treatment of Aggr6 cells with a selective potent inhibitor of PKA, dihydrochloride hydrate (H-89), abrogated the effects of both WNT5A (*5*) and Bt_2cAMP (*3*), proving that WNT5A stimulates the phosphorylation of CREB and ATF1 through the activation of the PKA pathway. The addition of H-89 alone (*6*) and in combination with WNT5A (*5*) or Bt_2cAMP (*3*) reduced the phosphoylation status of CREB and ATF1 slightly below the level found in untreated cells (*1*), demonstrating a certain baseline activity of the PKA pathway in Aggr6 cells.

In contrast, a pronounced activation of CREB and ATF1 could not be observed in the cell cultures of normal fibroblasts NHDF and Aggr2, neither through recombinant WNT5A (NHDF: *9*, Aggr2: *12*), nor after the addition of Bt_2cAMP (NHDF: *8*, Aggr2:

11), in comparison to untreated cells (NHDF: *7*, Aggr2: *10*), suggesting an impaired functional linkage between PKA and CREB/ATF1 in these cells.

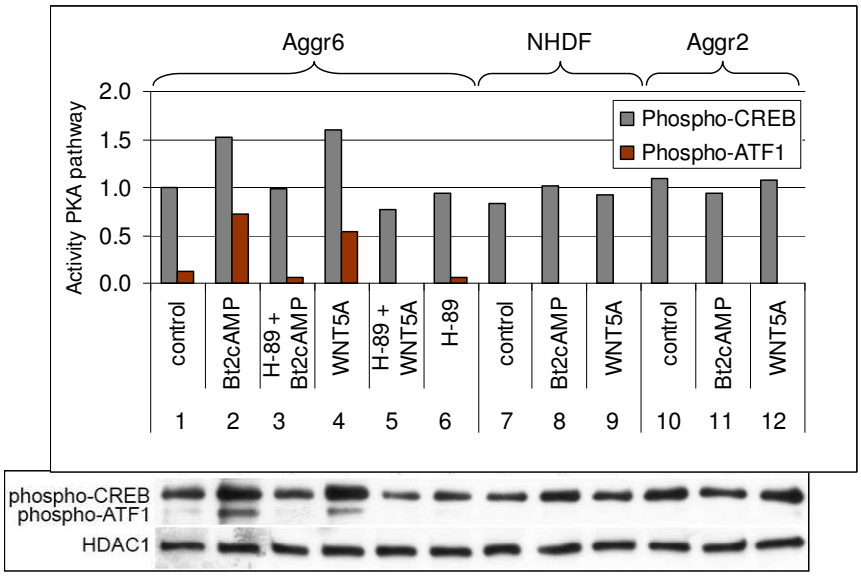

Figure 50: The impact of recombinant WNT5A on the activity of the PKA pathway. The activity of the PKA pathway was measured by Western blot for active, phospho-CREB and phospho-ATF1 in Aggr6 tumor cells (Aggr6) and normal human fibroblasts (NHDF, Aggr2). The different treatments are indicated. Control: untreated cells. The intensity of the bands below was quantified and the resulting numbers depicted as columns in the upper diagram. HDAC1: loading control.

- Analysis of the WNT5A-induced crosstalk between the PKA and the canonical Wnt pathway in Aggr6 cells

In the last section, WNT5A was shown to activate the PKA pathway specifically in the tumor cell culture Aggr6. In a next step it should be investigated, whether there is a crosstalk between the WNT5A-induced PKA and canonical Wnt signalling pathways. Aggr6 cells were treated with recombinant WNT5A alone or with WNT5A after a pre-treatment with the PKA-inhibitor H-89 to selectively inhibit its impact on the PKA pathway. Subsequently, the activity of the canonical Wnt pathway was measured both with Luciferase-assay (*Figure 51, L*) as well as with Western blot (*Figure 51, W*), using cytoplasmic protein extracts and an antibody against active, dephosphorylated β-catenin. Using both techniques, WNT5A was shown to increase the canonical Wnt signalling pathway activity (*4*) as compared to the activity in untreated cells (*1*). A pre-treatment with the PKA-inhibitor H-89, whereas completely abolishing the effect of WNT5A on the PKA pathway (*Figure 50*), did not modulate WNT5A's inducing impact on the canonical Wnt pathway (*Figure 51, 5*), demonstrating a lacking crosstalk between these two signalling pathways. This finding was further confirmed by the results obtained when treating the cells with the PKA pathway inducer Bt_2cAMP (*2*) or its inhibitor H-89 (*6*). Neither of the two had any effect on the canonical Wnt signalling pathway as compared to the activity in untreated cells (*1*). Consequently, the same is true for combined treatment with both inducer and inhibitor (*3*).

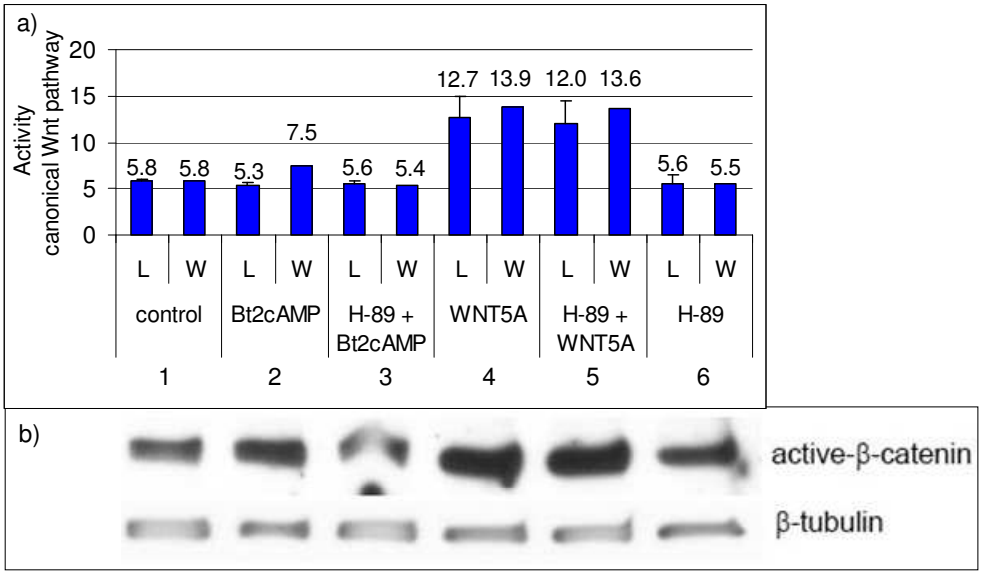

Figure 51: The analysis of the WNT5A-induced crosstalk between the PKA and the canonical Wnt pathway in Aggr6 cells.

a) The activity of the canonical Wnt signalling was measured by Luciferase assays (L; mean and standard deviation of three independent experiments) and Western blot (W) in Aggr6 tumor cells. The different treatments are indicated. Control: untreated cells.

b) Western blot for active β-catenin, aligned to *Figure 51 a).* The intensity of the bands was quantified and the resulting numbers integrated as columns (W) into *Figure 51 a).* β-tubulin: loading control.

4.2.5. The effect of WNT5A stimulation on the STAT signalling pathway

To determine, whether WNT5A signals via the STAT pathway, the activities of the downstream transcription factors STAT1α and STAT3 were compared between WNT5A-treated and untreated Aggr6 cells. As *Figure 52* demonstrates, WNT5A does not influence the activity of this pathway in Aggr6 cells.

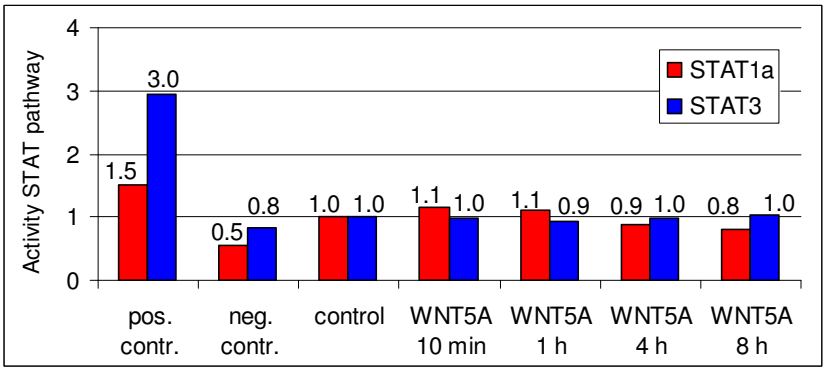

Figure 52: The impact of recombinant WNT5A on the activity of the STAT pathway in Aggr6 cells, determined using transcription factor-activity assays from Active Motif for STAT1α and STAT3. Cells were treated with recombinant WNT5A for the indicated periods of time. As a positive control, a nuclear protein extract of prolactin stimulated Nb2 cells (rat lymphoma cell line) was used for both antibodies, whereas the values for the negative controls are derived from wells containing no nuclear protein extract. Control: untreated cells.

4.2.6. Summary of the signalling pathway analyses

Figure 53 summarizes the results obtained in *Sections 4.2.1. – 4.2.5.* In the aggressive fibromatosis derived primary tumor cell culture Aggr6, recombinant WNT5A elicits the activity of the canonical Wnt, the Wnt-PCP, and the PKA pathway. No crosstalks between the latter two and the canonical Wnt pathway could be observed. In contrast, cell cultures of normal fibroblasts NHDF and Aggr2 show no signalling response in these pathways after stimulation with recombinant WNT5A.

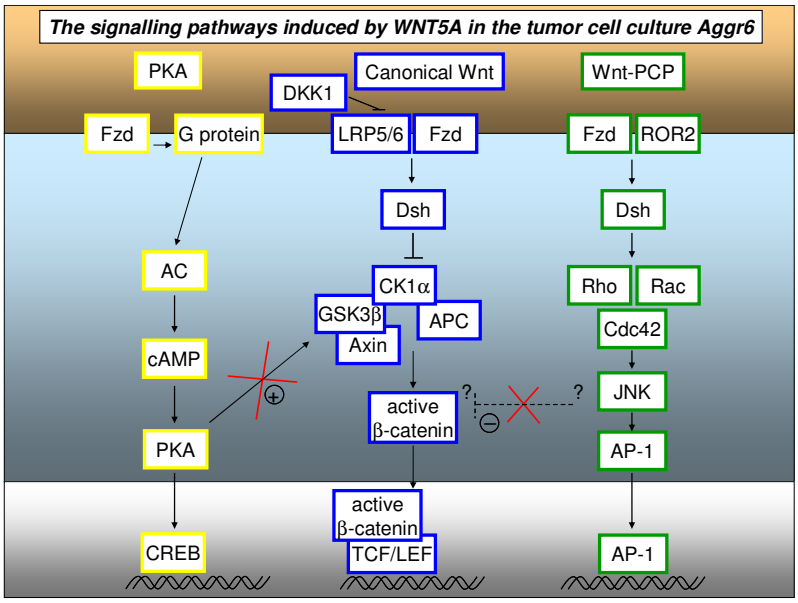

Figure 53: WNT5A activates the canonical Wnt, the Wnt-PCP and the PKA pathway in the aggressive fibromatosis derived tumor cell culture Aggr6. No crosstalks between the pathways could be observed.

4.3. The impact of WNT5A stimulation on proliferation and invasive behaviour of Aggr6 cells and cell cultures of normal fibroblasts

4.3.1. Proliferation

Figure 54 demonstrates that treatment of Aggr6 cells and cell cultures of normal fibroblasts Aggr2, NHDF and HDF15 with recombinant WNT5A led to an increase of the proliferation rate in Aggr6, whereas the other cell cultures remain unaffected.

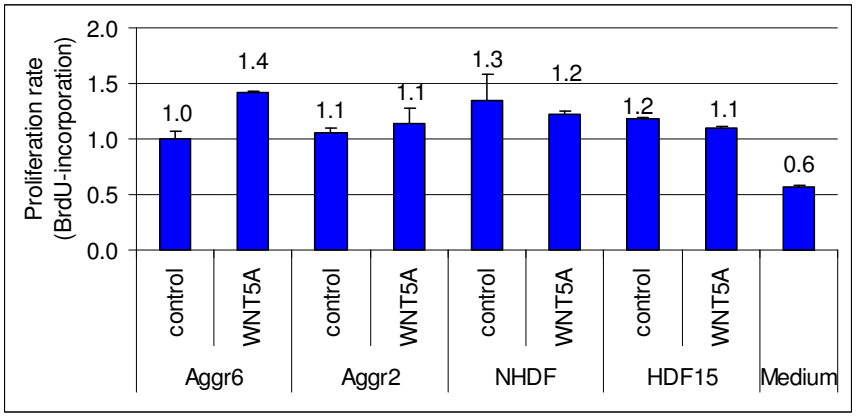

Figure 54: The impact of WNT5A on the proliferation rate in Aggr6 cells and cell cultures of normal fibroblasts Aggr2, NHDF and HDF15, measured using a BrdU-incorporation assay. Cells were treated with recombinant WNT5A for 48 hours. Measurements were performed in triplicates and demonstrated in the diagram as mean value and standard deviation. Control: untreated cells. Medium: negative control (the background signal obtained from wells containing growth medium but no cells).

To demonstrate the specificity of the observed effect in Aggr6, cells were simultaneously treated with recombinant WNT5A and a specific antibody against this protein. Doing so, the proliferation stimulating impact of WNT5A could mostly be abrogated (*Figure 55*).

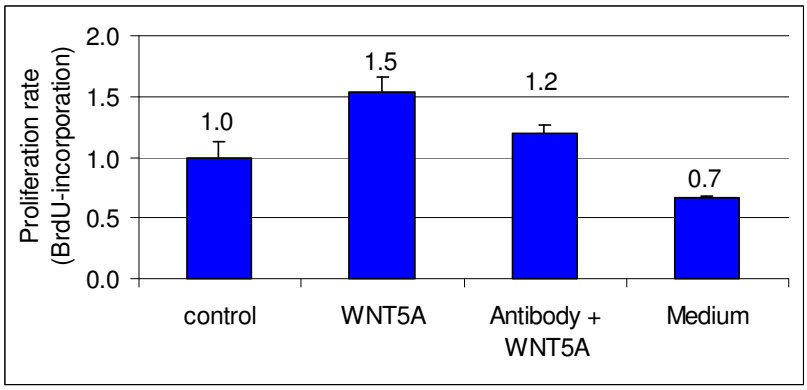

Figure 55: The specificity of the proliferation inducing effect of WNT5A in Aggr6 cells, determined using a BrdU-incorporation assay. Cells were treated with recombinant WNT5A without (WNT5A) or with the addition of its specific antibody (Antibody+WNT5A) for 48 hours. Measurements were performed in triplicates and demonstrated in the diagram as mean value and standard deviation. Control: untreated cells. Medium: negative control (the background signal obtained from wells containing growth medium but no cells).

Analyses summarized in S*ection 4.2.6.* revealed that recombinant WNT5A induces in Aggr6 cells the activity of three different pathways: the canonical Wnt, the Wnt-PCP, and the PKA pathway. Therefore, the following experiment was intended to explore, which of these three pathways are involved in the regulation of proliferation of Aggr6 cells. The cells were treated with WNT5A alone or in combination with a specific inhibitor of the particular pathway: DKK1 for the canonical Wnt, JNKI1 for the Wnt-PCP and H-89 for the PKA pathway. In addition, these inhibitors were added to the cells alone to investigate whether the activities of the corresponding pathways are relevant for the baseline proliferation of Aggr6. WNT5A proved again to be able to increase the proliferation rate of Aggr6 cells (*Figure 56, 2*) as compared to untreated cells (*1*). Whereas the inhibitor of the canonical Wnt (DKK1, *3*) and the Wnt-PCP pathway (JNKI1, *4*) alone did not influence the proliferation rate of Aggr6 cells, the inhibitor of the PKA pathway (H-89, *5*) reduced the emitted signal near to the level of the background signal of the medium (*10*). This finding demonstrates that an active PKA signalling pathway is essential for the baseline proliferation activity of Aggr6 cells. Whereas the specific inhibition of the canonical Wnt pathway (DKK1+WNT5A, *6*) and the Wnt-PCP pathway (JNKI1+WNT5A, *7*) did not impair WNT5A's positive effect on cellular proliferation, the abrogation of WNT5A's impact on the PKA pathway (H-89+WNT5A, *8*) almost completely abolished the proliferation activity of Aggr6 cells. Consequently, the combined treatment with the three inhibitors and recombinant WNT5A (*9*) led to a similar outcome. Thus, of the three pathways known to be activated by WNT5A, the PKA pathway elucidated to be the only one decisively involved in the regulation of proliferation of Aggr6 cells.

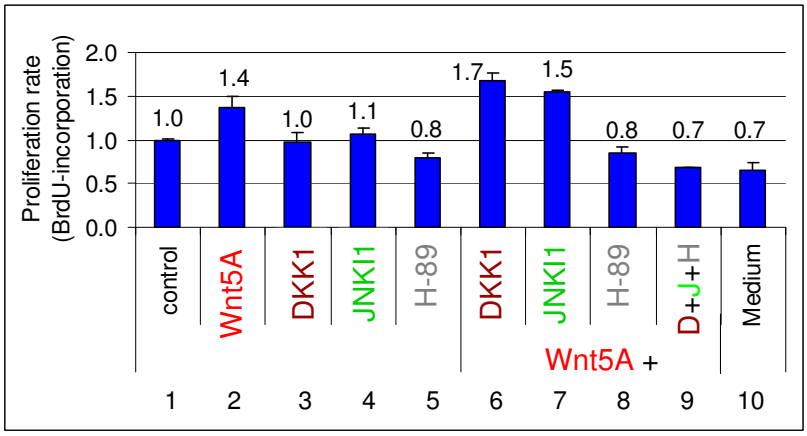

Figure 56: Analysis of the involvement of the three WNT5A-induced pathways in the regulation of proliferation of Aggr6 cells using a BrdU-incorporation assay. The cells were treated for 48 hours with the indicated compounds. Measurements were performed in triplicates and demonstrated in the diagram as mean value and standard deviation. Control: untreated cells. Medium: negative control (the background signal obtained from wells containing growth medium but no cells).

4.3.2. Invasion

For the analysis of the invasive behaviour of the cell cultures Aggr6, Aggr2 and NHDF in response to WNT5A, a type I collagen cell invasion assay was used. The results are depicted in *Figure 57*. If attracting the cells with 1% FCS in the bottom chambers (*A, D, G*), the cells do not invade at all, reflected by the emitted fluorescence signals that were equal to the ones obtained from the negative control wells (K) containing no cells. The invasive behaviour of all three cell cultures then steadily increased with higher concentrations of serum in the media of the bottom chambers (10% FCS: *B, E, H* vs. 20% FCS: *C, F, I*), since the emitted fluorescence signals in the corresponding wells increasingly differed from the ones in the negative control wells (10% FCS: *L*, 20% FCS: *M*). Whereas Aggr6 and Aggr2 cells showed overall a very similar invasive behaviour in response to serum attraction, NHDF cells proved to be more responsive, since their emitted fluorescence signals in the wells containing 10% (*H*) and 20% FCS (*I*) were above the levels emitted in the corresponding wells of Aggr6 (10% FCS: *B*, 20% FCS: *C*) and Aggr2 cells (10% FCS: *E*, 20% FCS: *F*). For Agg6, Aggr2 and NHDF cells, the addition of different concentrations of WNT5A (300 ng/ml vs. 600 ng/ml) into the media of both the inserts and the bottom chambers did not influence their invasive behaviour.

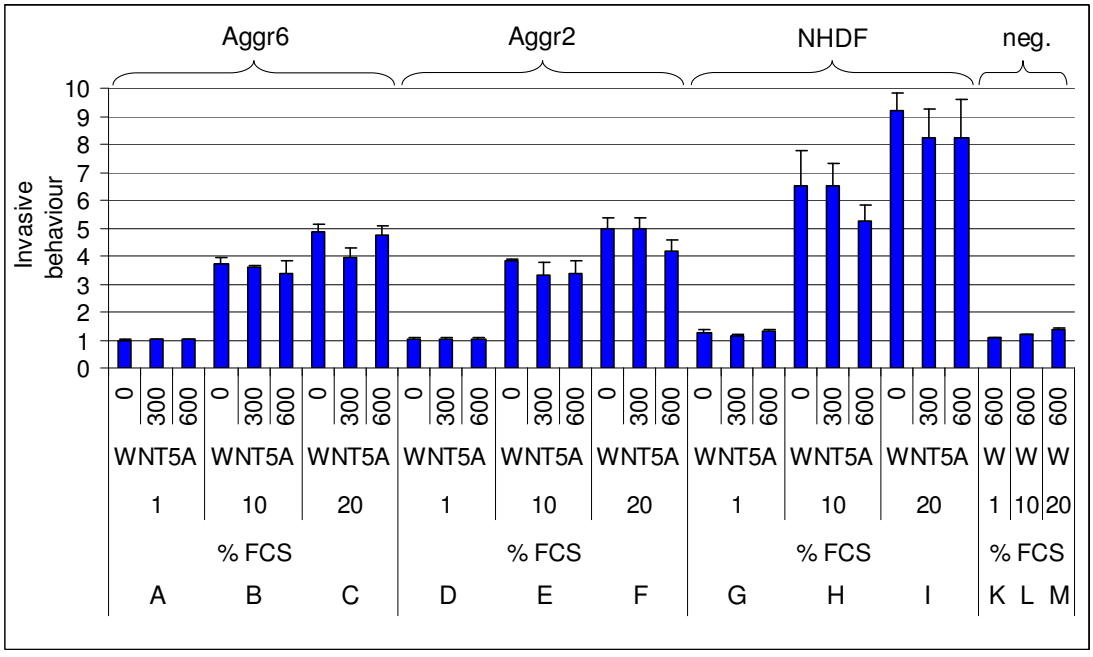

Figure 57: Invasive behaviour of the cell cultures Aggr6, Aggr2 and NHDF in response to serum attraction (1% vs. 10% vs. 20% FCS containing media in the bottom chambers) and addition of recombinant WNT5A (300ng/ml vs. 600ng/ml in both the inserts and the bottom chambers), analyzed using a type I collagen cell invasion assay. Cells were allowed to invade for 48 hours. Measurements were performed in triplicates and demonstrated in the diagram as mean value and standard deviation. neg.: negative control (the background signals obtained from wells containing growth medium, but no cells). W: WNT5A.

4.4. The differential gene expression induced by WNT5A stimulation in Aggr6 cells and cell cultures of normal fibroblasts

Agilent microarray analyses were performed to study the differential gene expression induced by recombinant WNT5A stimulation in the tumor cell culture Aggr6 and the cell cultures of normal fibroblasts Aggr2, Aggr4 and NHDF.

4.4.1. Selection of differentially expressed sequences to be further analyzed

Starting with 41'000 sequences present on each Agilent array, the following selection criteria were applied to reduce to number of sequences to be further analyzed: a fold-change of at least two, a p-value of less than 0.01 and an absolute expression level of more than 0.1 in at least one sample to be compared. Applying those selection criteria, the remaining numbers of differentially expressed sequences in the four cell cultures reflected the findings obtained in S*ections 4.2.* and *4.3.* concerning a selective responsiveness of the tumor cell culture Aggr6 towards recombinant WNT5A: it exerted a pronounced effect on the gene expression in Aggr6 cells, but hardly effected the cell cultures of normal fibroblasts Aggr2, Aggr4 and NHDF (*Figure 58*).

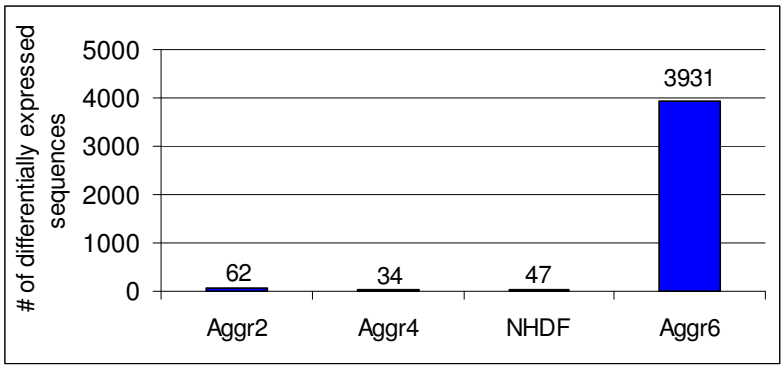

Figure 58: Numbers of differentially expressed sequences in the tumor cell culture Aggr6 and the cell cultures of normal fibroblasts Aggr2, Aggr4 and NHDF (Agilent microarray analysis).

4.4.2. Functional annotation of the selected sequences

For functional annotation of the selected 3'931 sequences found to be differentially expressed in Aggr6 cells after WNT5A stimulation, the biostatistical software GeneGo Metacore was used. Results in previous S*ections 4.2.* and *4.3.1.* demonstrated that WNT5A exerts a stimulating impact on signalling pathways and proliferation in Aggr6 cells. Therefore, the focus of the analysis was laid on genes involved in the biological processes cell signalling cascades and proliferation. In addition, genes coding for components of the extracellular matrix (ECM) and for proteins involved in cell-cell and cell-ECM adhesion were also selected, due to their relevance in the pathogenesis of aggressive fibromatoses (*Chapter I, Sections 2.1. and 4.4.1.*). *Figure 59* summarizes the numbers of genes belonging to these 3 biological processes. In addition, two examples are given.

	# of genes	upregulated	Examples	downregulated	Examples
Proliferation	184	127	CCND1, MYC	57	IGF1R, E2F6
Signalling pathway components	76	42	SMAD3, MAP3K5	34	CSNK1D, NR4A2
ECM / Cell adhesion	69	25	ITGA6, MMP3	44	ADAMTS1, HAS1

Figure 59: Numbers of genes belonging to the biological processes proliferation, signalling pathway components, and ECM/cell adhesion found to be differentially expressed in Aggr6 cells after the addition of WNT5A. Abbreviations: CCND1 = cyclin D1, MYC = c-myc, SMAD3 = mothers against decapentaplegic homolog (central transcription factor in TGFβ signalling pathway), MAP3K5 = mitogen-activated protein kinase kinase kinase 5, ITGA6 = integrin, alpha 6, MMP3 = matrix metalloproteinase 3, IGF1R = insulin-like growth factor 1 receptor, E2F6 = E2F transcription factor 6 (central role in the regulation of cell cycle), CSNK1D = casein kinase 1 delta, NR4A2 = nuclear receptor subfamily 4, group A, member 2 (member of the steroid-thyroid hormone-retinoid receptor superfamily), ADAMTS1 = ADAM metallopeptidase with thrombospondin type 1 motif, 1, HAS1 = hyaluronan synthase 1

Remarkable is the finding that 69% (127 out of 184) of the deregulated genes involved in the regulation of proliferation are induced in their expression after the addition of WNT5A. This indicates the prominent impact of WNT5A on the induction of proliferation in these cells. On the other hand, 64% (44/69) of the genes involved in ECM/Cell adhesion are downregulated in Aggr6 cells after treatment with WNT5A, suggesting that an overexpression of WNT5A in aggressive fibromatosis is not responsible for the observed accumulation of ECM proteins in this tumor.

The names, ratios, p-values and absolute expression levels of all genes listed in the figure above can be found in *Gene list E* in the *Appendix*.

4.5. Analysis of the reason for the differential responsiveness of tumor cells and normal fibroblasts towards WNT5A stimulation

Having shown that recombinant WNT5A activates the canonical Wnt, the Wnt-PCP and the PKA pathway (S*ection 4.2*) and stimulates proliferation (S*ection 4.3.1.*) in the tumor cell culture Aggr6, but not in the cell cultures of normal fibroblasts, the question raises what could be the reason for this discrepancy.

4.5.1. Analysis of Fzd receptor expression in tumor tissues and corresponding cell cultures

One possible explanation would be that Aggr6 cells express the relevant WNT5A receptors, whereas normal fibroblasts do not. Agilent microarray data revealed that all the aggressive fibromatosis tumor tissues analyzed in *Chapter I* (pool of 10 individual aggressive fibromatoses (Aggr pool) and 5 individually analyzed tumors Aggr1, Aggr2, Aggr4, Aggr6, Aggr7) overexpress the mRNAs coding for the Wnt receptors Fzd1, Fzd2, Fzd7, Fzd10 and ROR2 in comparison to the pool of reference fibrous tissues (*Figure 60 a*). Analysis of Agilent microarray data derived from cell culture RNA samples (*Chapter II*) revealed that of these five receptors, Fzd1, Fzd2 and Fzd7 (red in *Figure 60*) were also found to be overexpressed in the tumor cell culture Aggr6 as compared to the pool of normal human dermal fibroblasts, but mostly not in the other aggressive fibromatosis-derived cell cultures Aggr1, Aggr2, Aggr4 and Aggr7 (*Figure 60 b*).

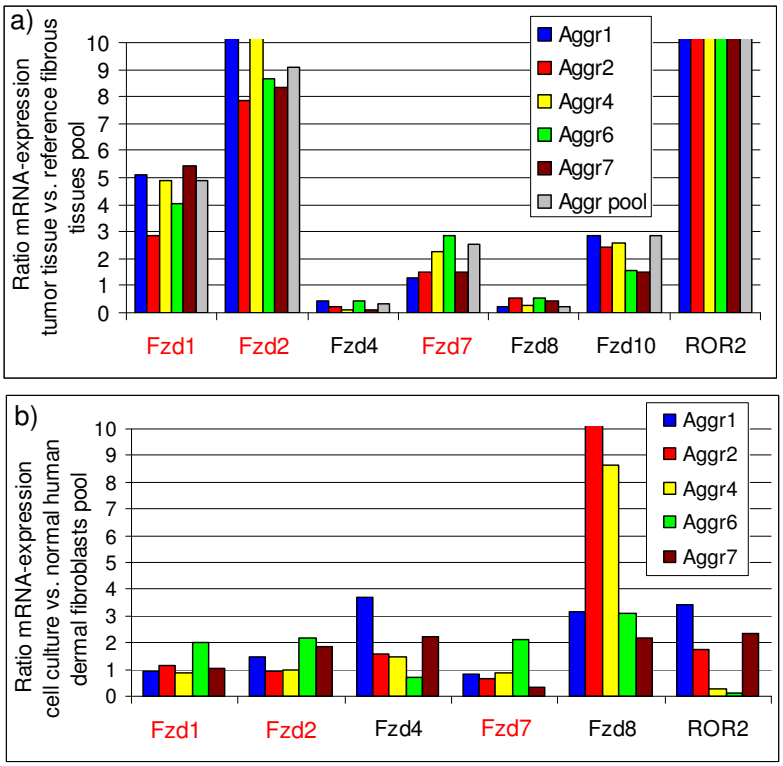

Figure 60: Ratios of Wnt receptor expressions in tissues of aggressive fibromatoses vs. pooled reference fibrous tissues (*a*) and in primary cell cultures of aggressive fibromatoses vs. pooled normal human dermal fibroblast cell cultures (*b*), determined using Agilent microarray analysis. Presented are those receptors that are differentially expressed in at least one comparison tumor tissue vs. reference pool and of primary cell culture vs. normal human dermal fibroblasts pool, respectively.

These findings suggest that aggressive fibromatosis tumor cells overexpress Fzd1, Fzd2 and Fzd7, what renders them responsive to the impact of WNT5A on cellular signalling pathways and proliferation.

4.5.2. Activation of the canonical Wnt pathway in cell cultures of normal fibroblasts

A possible reason for the higher expression of those Fzd receptors in the tumor cell culture Aggr6 may be the increased activity of the canonical Wnt signalling pathway as compared to the cell cultures of normal fibroblasts (*Figure 44*). A certain level of activity within this pathway may be a prerequisite for WNT5A to be able to execute its function, since it may lead to the expression of those receptors. To substantiate this theory, NHDF and Aggr2 cells were transfected with a plasmid expressing β-catenin carrying a deletion at codon 45 (Δ45-β-catenin). This mutation protects it from degradation by the β-catenin degradation complex and therefore leads to a constitutive activation of the canonical Wnt signalling pathway. *Figure 61* illustrates that an activation indeed occurred, since both cell cultures are characterized by increased canonical Wnt signalling on the Luciferase assay 24 hours (NHDF: *2*, Aggr2: *7*) and 48 hours (NHDF: *4*, Aggr2: *9*) after transfection, as compared to the activities in untransfected cells (NHDF: *1*, Aggr2: *6*). However, WNT5A was still not able to further activate this pathway, neither in the cell culture NHDF (24h: *3*, 48h: *5*), nor in Aggr2 cells (24h: *8*, 48h: *10*), demonstrating that the above presented theory did not prove to be true.

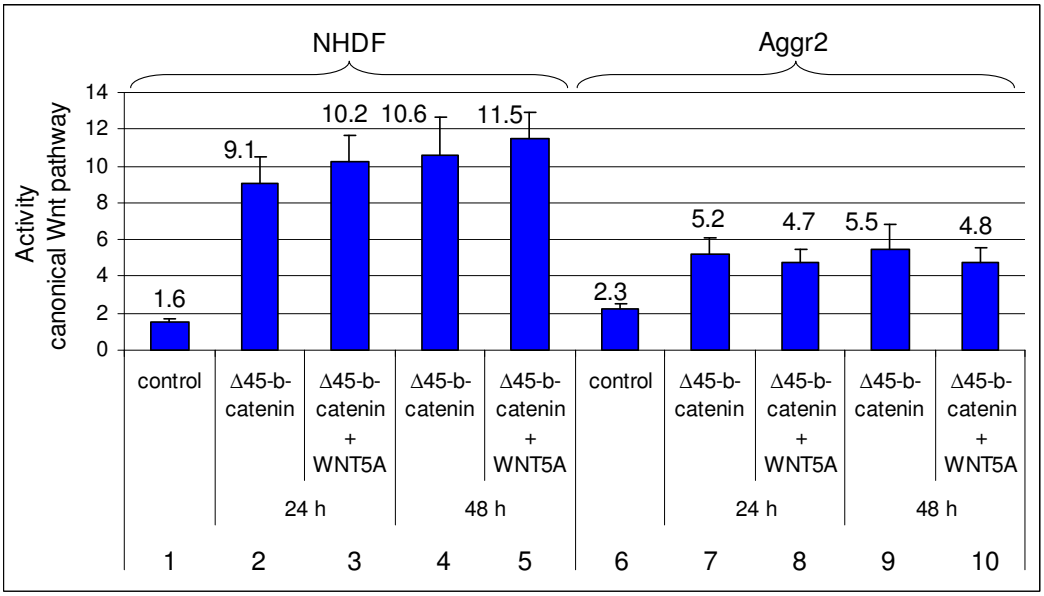

Figure 61: Activity of the canonical Wnt signalling pathway in cell cultures of normal fibroblasts NHDF and Aggr2 after transfection with a Δ45-β-catenin expression plasmid and subsequent (16 h or 40 h after transfection) treatment with 150ng/ml recombinant WNT5A for 8 hours (Luciferase assay). The mean and standard deviation of three independent tests is demonstrated. Control: untransfected and untreated cells.

4.5.3. Downregulation of Fzd1, Fzd2 and Fzd7 in the tumor cell culture Aggr6

To further investigate the functional relevance of the observed overexpression of Fzd1, Fzd2 and Fzd7 in Aggr6 cells, their expressions were inhibited by transfecting Aggr6 cells with different concentrations (20nM, 200nM, 2µM) of a pool of siRNAs directed against the mRNAs of those Fzd receptors. 16 hours, 40 hours and 64 hours after transfection, the cells were treated with recombinant WNT5A for 8 hours to explore whether its inducing impact on the canonical Wnt signalling pathway was abrogated or not (Luciferase assay). *Figure 62* illustrates that WNT5A's stimulating effect on the activity of the canonical Wnt signalling pathway in Aggr6 cells was not diminished by the transfection with siRNAs against Fzd1, Fzd2 and Fzd7.

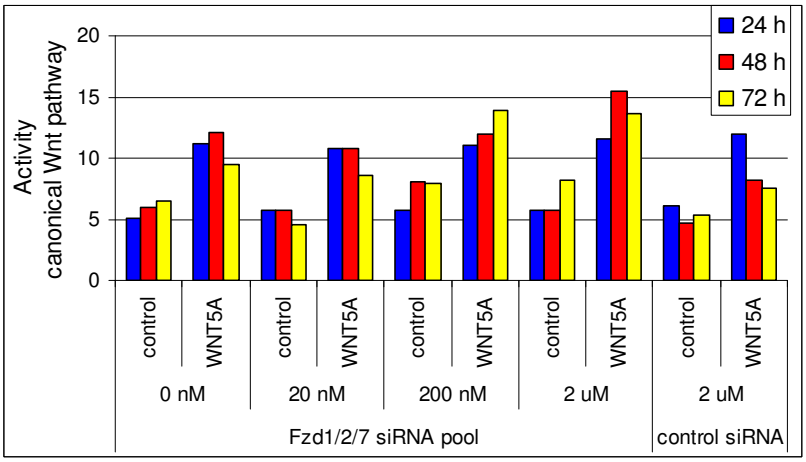

Figure 62: Activity of the canonical Wnt signalling pathway in Aggr6 cells after transfection with a pool of siRNAs against the Wnt receptors Fzd1, Fzd2 and Fzd7 (concentrations: 20 nM vs. 200 nM vs. 2 µM) and subsequent (16 h, 40 h, and 64 h after transfection) treatment with 150ng/ml recombinant WNT5A for 8 hours, determined using Luciferase assay. Control: untreated cells. control siRNA: negative control (a pool of four non-specified, non-targeting siRNAs)

5. Discussion

5.1. Summary of the results

Agg6 tumor cells carrying a monoallelic β-catenin mutation at codon serine 45 (S45F) are characterized by an enhanced activity of the canonical Wnt signalling pathway as compared to cell cultures of normal fibroblasts. This is in agreement with the classical theory whereupon a monoallelic S45F mutation impedes phosphorylation at this residue by CK1α and subsequently also the phosphorylation at the other three residues by GSK3β, leading to the accumulation of dephosphorylated, active β-catenin.

There is a measurable effect of endogenously produced Wnt ligands on the canonical Wnt signalling pathway activity in Aggr6 cells via wildtype β-catenin, contributing to the impact of mutated β-catenin on this pathway.

Whereas the endogenously produced WNT5A exerted no measurable effect on the canonical Wnt signalling pathway activity in Aggr6 tumor cells, recombinant WNT5A had an inducing impact on this pathway, because it could be abrogated by the simultaneous addition of the canonical Wnt inhibitor DKK1. The observed induction was specific, since the concomitant treatment with anti-WNT5A antibodies abolished the effect of recombinant WNT5A. In contrast to the results obtained for Aggr6 cells, normal fibroblasts did not show any increase in the activity of the canonical Wnt signalling pathway after WNT5A stimulation.

Recombinant WNT5A activates the non-canonical Wnt-PCP and PKA pathways in Aggr6 tumor cells as well, whereas a crosstalk of those pathways to the canonical Wnt signalling cascade could not be observed. Cell cultures of normal fibroblasts did not respond to the addition of WNT5A with an increased activity of the Wnt-PCP or PKA pathway. The non-canonical Wnt-Ca^{2+} pathway as well as the STAT signalling pathway were not affected by the addition of WNT5A.

A cell proliferation assay revealed that recombinant WNT5A selectively increased the proliferation rate of the tumor cell culture Aggr6. Whereas the specific inhibition of the canonical Wnt pathway and the Wnt-PCP pathway did not impair WNT5A's positive effect on cellular proliferation, the abrogation of WNT5A's impact on the PKA pathway almost completely abolished the proliferative activity of Aggr6 cells. Thus, of the three pathways known to be activated by WNT5A, the PKA pathway elucidated to be the only one decisively involved in the regulation of proliferation of Aggr6 tumor cells.

For the analysis of the invasive behaviour of the tumor cell culture Aggr6 and the cell cultures of normal fibroblasts Aggr2 and NHDF in response to WNT5A, a type I collagen cell invasion assay was used. Neither of them responded to the addition of recombinant WNT5A with an altered invasive behaviour.

The numbers of differentially expressed sequences obtained by a microarray experiment reflected the above findings concerning a selective responsiveness of the tumor cell culture Aggr6 towards recombinant WNT5A: it exerted a pronounced effect

on the gene expression in Aggr6 cells, but hardly affected the cell cultures of normal fibroblasts Aggr2, Aggr4 and NHDF.

Fzd1, Fzd2 and Fzd7 receptors were found to be overexpressed in the tumor cell culture Aggr6 as compared to the pool of normal human dermal fibroblasts, but mostly not in the other aggressive fibromatosis-derived cell cultures Aggr1, Aggr2, Aggr4 and Aggr7. Those Fzd receptors are also upregulated in aggressive fibromatosis tumor tissues in comparison to the reference fibrous tissues, providing a possible explanation for the selective responsiveness of Aggr6 tumor cells towards recombinant WNT5A. However, WNT5A's stimulating effect on the activity of the canonical Wnt signalling pathway in Aggr6 cells could not abrgated by the transfection with siRNAs against Fzd1, Fzd2 and Fzd7.

By the transfection of a β-catenin expression plasmid into cell cultures of normal fibroblast, the activity of the canonical Wnt pathway could be enhanced in normal fibroblasts, simulating the situation in Aggr6 tumor cells. However, recombinant WNT5A was still not able to further activate this pathway, demonstrating that an activated canonical Wnt pathway is not responsible for an overexpression of relevant WNT5A receptors.

5.2. Analysis of the performance of subcellular protein fractionation

Western blot analyses presented in *Section 4.1.* were aimed to reveal the accuracy of the subcellular protein fractionation process performed by the Nuclear Extract Kit from Active Motif, since these protein extracts represent the basis for the cell signalling activity experiments depicted in *Section 4.2.*

The soluble nuclear protein fraction that was used for the transcription factor-activity assays and the CREB Western blot experiment was shown to be free from any contaminations derived from the other protein fractions, therefore represents a trustable basis for those experiments. In addition a substantial contamination of the soluble cytoplasmic protein fraction, used for β-catenin Western blot analyses, by soluble nuclear proteins could be excluded as well, demonstrating its justified use for those studies.

5.3. Analysis of the impact of WNT5A on cellular signalling pathways in Aggr6 and cell cultures of normal fibroblasts

5.3.1. The effect of WNT5A stimulation on the canonical Wnt signalling pathway

Our observation, that the aggressive fibromatosis tumor cell culture Aggr6 is characterized by an increased endogenous activity of the canonical Wnt signalling pathway as compared to the activity in normal fibroblast cell cultures, is in agreement with findings published in the literature reporting the same (Amini Nik *et al.*, 2005; Denys *et al.*, 2004; Tejpar *et al.*, 2001) and also confirmes the prevalent theory whereupon a monoallelic mutation at serine 45 makes impossible a phosphorylation at this residue by CK1α and subsequently impedes the phosphorylation at the other

three residues by GSK3β, leading to the accumulation of dephosphorylated, active β-catenin (Amit *et al.*, 2002; Liu *et al.*, 2002; Yanagawa *et al.*, 2002).

In the tumor cell culture Aggr6, the enhanced activity of the canonical Wnt signalling pathway was shown to be derived from both an activating mutation in the oncogene β-catenin and an endogenous stimulation through secreted Wnt ligands. A finding, that have not been described for tumor cell cultures of aggressive fibrombatoses, yet.

It was also observed for the first time, that cell cultures of normal fibroblasts derived from aggressive fibromatoses are characterized by a higher activity of the canonical Wnt signalling pathway in comparison to normal human dermal fibroblast cultures, pointing towards the existence of an endogenous stimulation through secreted Wnt ligands in these cell cultures as well.

Whereas endogenously produced WNT5A does not exert any impact on the canonical Wnt pathway activity in Aggr6 cells, recombinant WNT5A was shown to selectively induce the activity of this pathway in the tumor cell culture Aggr6, whereas all the cell cultures of normal fibroblasts did not respond. As pointed out in Chapter II, section 4.3, the amounts of WNT5A used for stimulation were 15 times higher than the quantity of endogenous WNT5A present in the culture supernatant of Aggr 6 cells. Historically, WNT5A was categorized as a 'non-canonical' Wnt ligand, since it was not able to induce a secondary axis in Xenopus, or to transform mouse mammary epithelial C57MG cells (Du *et al.*, 1995; Wong *et al.*, 1994), both processes depending on the activity of the canonical Wnt pathway. But meanwhile, this strict categorization has been abandoned after the observations that WNT5A can activate the canonical Wnt pathway as well, depending on the context of Fzd receptors expressed (Deardorff *et al.*, 1998; He *et al.*, 1997; Itoh *et al.*, 1998; Mikels and Nusse, 2006; Umbhauer *et al.*, 2000; Yang *et al.*, 2009). However, in context of tumorigenesis, a contribution of WNT5A to the pathogenesis through the activation of the canonical Wnt pathway has been published only once, so far. In a study done in the human immortalized mammary epithelial cell line HC11, WNT5A was shown to increase the proliferation rate by transactivating the receptor tyrosine kinase EGFR through the canonical Wnt-pathway leading to the expression of matrix metalloproteinases (MMPs) and the resultant increase of the availability of EGFR ligands (Civenni *et al.*, 2003). All other functional studies performed to reveal its impact on the pathogenesis a diverse array of cancers came to the conclusion that it signals via a non-canonical pathway (*Section 1.2.1.*).

5.3.2. The impact of recombinant WNT5A on the Wnt-Ca^{2+} pathway in Aggr6 cells

NFAT is a transcription factor downstream of calmodulin (CaM) and the protein phosphatase calcineurin (PP2B) and has been shown to be involved in the Wnt-Ca^{2+} pathway (Saneyoshi *et al.*, 2002). In Aggr6 cells, a transcription factor-activity assay for the family member NFATC1 (NFAT2) revealed that recombinant WNT5A does not lead to its dephosphorylation and activation, indicating that it does not signal through the Wnt-Ca^{2+} pathway.

In the literature, several publications report about the relevance of a WNT5A-induced Wnt-Ca^{2+} pathway activity in the pathogenesis of different types of cancer. Via this pathway, it exerts tumor-suppressing activities in breast cancer (Dejmek *et al.*, 2006), hematopoietic neoplasia (Liang *et al.*, 2003), and neuroblastoma (Blanc *et al.*, 2005),

whereas tumor-promoting abilities in cutaneous melanoma (Dissanayake *et al.*, 2008; O'Connell *et al.*, 2009b; Weeraratna *et al.*, 2002; Witze *et al.*, 2008) and gastric cancer (Kurayoshi *et al.*, 2006; Yamamoto *et al.*, 2009).

Additionally, a study using mouse B-cells reports about an inhibitory effect of WNT5A on the canonical Wnt signalling pathway and cellular proliferation via the activation of the Wnt-Ca^{2+}-pathway (Liang *et al.*, 2003).

5.3.3. The effect of WNT5A stimulation on the Wnt-PCP signalling pathway

Transcription factor activity assays for c-jun, junB and junD, in combination with a pre-treatment of the cells with a specific JNK-inhibitor, JNKI1, led to the conclusion that WNT5A selectively activates the Wnt-PCP pathway in the tumor cell culture Aggr6, but not in the cultures of normal fibroblasts.

In the literature, an activation of this pathway by WNT5A has been brought into context with an increased invasive capacity of the human breast cancer cell line MCF-7 (Pukrop *et al.*, 2006), an increased migration and invasion behaviour of human cutaneous melanoma (O'Connell *et al.*, 2009a; Weeraratna *et al.*, 2002; Witze *et al.*, 2008) and osteosarcoma cell cultures (Enomoto *et al.*, 2009) and an increased number of liver metastases after injection of human gastric cancer cells into the spleens of nude mice (Yamamoto *et al.*, 2009). Therefore, the observed overexpression of WNT5A in aggressive fibromatoses may contribute to their characteristic invasive growth behaviour through the activation the Wnt-PCP pathway. However, the addition of recombinant WNT5A to Aggr6 cells did not improve their invasive capacity (*Section 4.3.2.*), suggesting that in aggressive fibromatoses other mechanisms are responsible for this behaviour.

In a study using human cholangiocarcinoma cells, WNT5A was reported to inhibit their proliferation by the activation of the Wnt-PCP pathway (DeMorrow *et al.*, 2008), a finding that contrasts our results in Aggr6 cells, whereupon WNT5A exerts no influence on cellular proliferation via the Wnt-PCP pathway (*Section 4.3.1.*).

In addition, two reports using human osteosarcoma cell lines (Enomoto *et al.*, 2009), and human colon carcinoma cell lines (MacLeod *et al.*, 2007) describe an inhibition of the canonical Wnt pathway through the activation of the Wnt-PCP pathway by WNT5A, leading to increased invasion and migration of the osteosarcoma cells (Enomoto *et al.*, 2009). In contrast to the results of these studies, our data in aggressive fibromatosis tumor cells Aggr6 did not show up any crosstalk between these two WNT5A-induced pathways.

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5.3.4. The effect of WNT5A stimulation on the PKA signalling pathway

In agreement with results of a study based on the human breast cancer cell line MCF-7 (Hansen *et al.*, 2009), our study revealed that WNT5A activates in Aggr6 cells the transcription factor CREB in a PKA-dependent manner, indicating that it activates the PKA pathway in these cells. In contrast, cell cultures of normal fibroblasts did not respond to the addition of recombinant WNT5A with an increased phosphorylation of CREB. Hansen's study is the only one so far published in the literature describing an activation of the PKA pathway by WNT5A in cancerous cells. The authors of another publication (Torii *et al.*, 2008) worked with primary human dermal fibroblast cultures

and report their reduced serum starvation induced apoptosis after WNT5A-dependent activation of the PKA pathway. In our study, fibroblast cell cultures, including the normal dermal fibroblast cell culture NHDF, did not show an increased activation of CREB, neither after WNT5A-treatment, nor the addition of the PKA-inducer Bt_2cAMP, indicating that there is no functional linkage between PKA and CREB in these cells. This discrepancy might be due to the fact that the fibroblasts in Torii's study were cultivated in serum-free medium prior to their treatment with WNT5A. Under serum-starvation conditions, they report also a crosstalk between PKA and GSK3β, leading to its Ser9-phosphorylation-dependent inhibition and subsequent activation of TCF-dependent transcription. The addition of WNT5A to Aggr6 cells after their pre-treatment with the PKA-inhibitor H-89 did not influence the observed stimulating effect of WNT5A on the canonical Wnt pathway, demonstrating that under the growth conditions applied in our study, there is no crosstalk between the PKA and the canonical Wnt signalling pathway.

5.3.5. The effect of WNT5A stimulation on the STAT signalling pathway

In human cutaneous melanoma cell cultures, WNT5A was reported to activate the transcription factor STAT3 in a PKC-dependent manner leading to a suppressed expression of tumor-associated antigens (Dissanayake *et al.*, 2008). Our approach to study in Aggr6 cells the impact of WNT5A on the activation of STAT1α and STAT3 using transcription factor activity assays did not reveal an influence of WNT5A on the activity of the STAT pathway in these cells.

5.4. The impact of WNT5A stimulation on proliferation and invasive behaviour of Aggr6 cells and cell cultures of normal fibroblasts

5.4.1. Proliferation

The BrdU-proliferation assay demonstrated that Aggr6 cells respond to the addition of recombinant WNT5A with an increased proliferation rate, whereas the cell cultures of normal fibroblasts Aggr2, NHDF and HDF15 did not respond.

A positive effect of WNT5A on proliferation has been described in the literature in context with diverse types of human cell cultures, including immortalized mammary epithelial cells (Civenni *et al.*, 2003), cutaneous melanoma cells (Schwartz *et al.*, 2009), papillary thyroid cancer cells (McCall *et al.*, 2007), pancreatic cancer cells (Schwartz *et al.*, 2009), glioblastoma-derived tumor cells (Yu *et al.*, 2007), and non-small-cell lung cancer cells (Huang *et al.*, 2009), therefore demonstrating WNT5A's tumor-promoting effect in these types of cancers. But the exact mechanism by which WNT5A contributes to this effect, has not been investigated in all these studies, with the exception of the report by Civenni, describing an involvement of the canonical Wnt pathway.

With the aim to elucidate the mechanisms of the WNT5A-dependent proliferation stimulation in Aggr6, the cells were pre-treated with inhibitors of the three pathways known to be activated after WNT5A stimulation (canonical Wnt, Wnt-PCP and PKA). The results of these experiments revealed that the PKA pathway is the only one decisively involved in the regulation of proliferation of Aggr6 cells.

The PKA pathway has been reported to stimulate cell growth in many cell types while inhibiting it in others (Stork and Schmitt, 2002). It has been proposed, that PKA regulates cell proliferation through its phosphorylation and activation of myosine phosphatase targeting subunit 1 (MYPT1) and phosphorylation and inactivation of the tumor suppressor protein neurofibromin 2 (NF2, merlin), an important inhibitor of cell proliferation in many cell and tissue types (Alfthan *et al.*, 2004; Umeda *et al.*, 2008). On the other hand, MYPT1 was shown to dephosphorylate and activate NF2 itself (Jin *et al.*, 2006). Therefore, PKA exerts its proliferation inducing effect by directly inhibiting NF2 and its proliferation inhibiting effect by indirectly activating NF2 through MYPT1. The PKA-inhibitor H-89 was reported to inhibit cell proliferation in human cell cultures of hepatocellular carcinoma (HepG2), breast cancer (MCF-7), lung adenocarcinoma (A549) and squamous cell carcinoma (A431), findings that confirm our observation in Aggr6 cells. Knockdown of MYPT1 in the human cervical cancer cell line HeLa attenuated their H-89-induced cell growth inhibition, further suggesting that the phosphorylation level of NF2 regulated by both PKA and MYPT1 is critically involved in the regulation of cell proliferation (Umeda *et al.*, 2008). At higher concentrations (25µM), H-89 was reported to compromise the viability of HeLa cells, whereas at 10µM (the concentration that was used in our study) this effect was less pronounced, still allowing cell growth, but significantly retarded as compared to untreated cells (Umeda *et al.*, 2008).

In addition, PKA was shown to stimulate proliferation through the phosphorylation and activation of the MAP3K BRAF (v-raf murine sarcoma viral oncogene homolog B1), leading to the activation of MAPK-pathways, known to be involved in the regulation of cell proliferation (Chiaradonna *et al.*, 2008). CREB itself also influences proliferation, since among its targets, numerous cell cycle promoting genes can be found, including cyclins D1 (CCND1) and A2 (CCNA2), cyclin-dependent kinase 5 (CDK5), FBJ murine osteosarcoma viral oncogene homolog (FOS), fibroblast growth factor 6 (FGF6) and JUND, just to name a few (Mayr and Montminy, 2001).

That the activation of the canonical Wnt pathway does not contribute to the proliferation stimulating effect of WNT5A in Aggr6 cells is somehow surprising, considering the central role of this pathway in the pathogenesis of aggressive fibromatoses and other cancers (*Chapter I, Section 1.1.3.* and *2.2.1.*). In addition, it has been reported that primary cell cultures of aggressive fibromatoses derived from FAP-patients, therefore carrying biallelic inactivating mutations in the tumor suppressor gene APC and being characterized by an increased activity in the canonical Wnt pathway, show an increased proliferation rate in comparison to the same cultures ectopically expressing a wildtype APC-construct, and therefore being characterized by a reduced canonical Wnt signalling activity (Li *et al.*, 1998). However, the tumor cell culture Aggr6, that shows an increased endogenous canonical Wnt signalling pathway activity as compared to cell cultures of normal fibroblasts Aggr2, NHDF and HDF15, does not proliferate faster than those three cell cultures, confirming that in our cell cultures used and under our growth conditions applied, the canonical Wnt signalling pathway activity does not influence cellular proliferation.

5.4.2. Invasion

The type I collagen cell invasion assay demonstrated that neither the primary tumor cell culture Aggr6, nor Aggr2 or NHDF normal fibroblasts respond to the addition of recombinant WNT5A with an alteration of their invasive behaviour.

WNT5A was reported to promote the invasion of tumor cells in several types of cancer, including breast cancer (Pukrop *et al.*, 2006), cutaneous melanoma (O'Connell *et al.*, 2009b; Weeraratna *et al.*, 2002), osteosarcoma (Enomoto *et al.*, 2009), pancreatic cancer (Ripka *et al.*, 2007) and glioblastoma (Yu *et al.*, 2007). On the other hand, WNT5A was shown to inhibit the invasive behaviour of breast cancer (Dejmek *et al.*, 2006; Medrek *et al.*, 2009; Safholm *et al.*, 2008) and neuroblastoma (Blanc *et al.*, 2005) tumor cells.

5.5. The differential gene expression induced by WNT5A stimulation in Aggr6 cells and cell cultures of normal fibroblasts

The selective impact of WNT5A on the tumor cell culture Aggr6 is impressively demonstrated by looking at the simple numbers of sequences differentially expressed after WNT5A stimulation of Aggr6 cells and cell cultures of normal fibroblasts using Agilent microarray gene expression analysis. Whereas nearly 4'000 sequences were found to be differentially expressed in Aggr6 cells, not even 100 were regulated in Aggr2, Aggr4 and NHDF normal fibroblasts.

The functional annotation of the selected differentially expressed genes in Aggr6 identified 184 genes to be involved in the regulation of proliferation, whereas 127 of them (69%) were found to be induced in their expression after WNT5A-treatment. Just to name a few examples, the upregulated genes include cyclins such as A2, B1, B2, D1 and G2, growth factors such as fibroblast growth factor 2 (FGF2) or platelet-derived growth factor A (PDGFA) and oncogenes like c-myc and epidermal growth factor receptor (EGFR).

The other focus was laid on intracellular components of signalling pathways. Also in this biological process, a lot of genes (76) were found to be differentially expressed, including among the upregulated genes the transcription factors of the TGFβ signalling pathway SMAD1, SMAD3 and SMAD4. Therefore, an increased expression of WNT5A in aggressive fibromatosis may lead to a transactivation of the TGFβ signalling pathway through the upregulation of these three critical components. However, looking at the gene expression data presented in *Chapter I* reveals that those transcription factors are not upregulated in tissues of aggressive fibromatosis as compared to reference fibrous tissues. SMAD3 was even shown to be downregulated.

69 genes belonging to the biological processes extracellular matrix (ECM), cell-cell and cell-ECM adhesion were found to be regulated in their expressions by WNT5A stimulation. Only 25 (36%) of them were upregulated, lacking any collagens, demonstrating that an increased expression of WNT5A in aggressive fibromatosis does not contribute to the characteristic accumulation of ECM proteins (*Chapter I, Section 2.1.*).

5.6. Analysis of the reason for the differential responsiveness of tumor cells and normal fibroblasts towards WNT5A stimulation

The analyses of the WNT5A-reguated signalling pathways and cellular behaviour demonstrated the selective impact of this protein on the tumor cell culture Aggr6. The missing responsiveness of the cell cultures of normal fibroblasts led to the suspicion, that they may lack the relevant receptors. An analysis of microarray gene expression data performed both on tissues and primary cell cultures revealed that the tumor cell culture Aggr6 selectively overexpresses the Wnt receptors Fzd1, Fzd2 and Fzd7 as compared to primary cell cultures of normal fibroblasts, and that these receptors are also overexpressed in aggressive fibromatosis tumor tissues as compared to reference fibrous tissues.

A downregulation of those receptors by the means of siRNAs transfected into Aggr6 cells did not lead to the expected effect of an abrogated WNT5A impact on these cells. Because of the very low transfection efficiency of primary cell cultures of aggressive fibromatosis, that is in the range of 15% at best, a confirmation of a successful downregulation of the targets on the protein level without any previous cell sorting approach could not be performed. Therefore, we do not know whether an inhibition of the expressions of Fzd1, Fzd2 and Fzd7 in the transfected cells indeed occurred.

The other approach, transfecting cell cultures of normal fibroblasts Aggr2 and NHDF with a Δ45-β-catenin expression plasmid, did not induce the expression of those Fzd receptors.. Although an increase of TCF-dependent transcription both in Aggr2 and NHDF could be observed, recombinant WNT5A was still not able to further activate it.

In conclusion, WNT5A activates the canonical Wnt as well as the non-canonical Wnt-PCP and PKA pathways. No crosstalk between the latter and the canonical pathway coud be observed. WNT5A stimulates the proliferation in a PKA pathway dependent manner, but does not affect the invasive behaviour of Aggr6 cells. In an attempt to investigarte the reason for the differential responsiveness of tumor cells and normal fibroblasts towards recombinant WNT5A, candidate Fzd receptors were repressed in their expressions by siRNA. This did not abolish WNT5A's effect on the activation of the canonical Wnt pathway. In order to investigate the possibility of an induction of relevant Fzd receptors by an active canonical Wnt pathway, normal fibroblasts were transfected with a β-catenin expression plasmid. Although an increased pathway activity could be observed, WNT5A was still not able to exert its stimulating effect.

Therefore, the mechanisms being responsible for the observed discrepancy between Aggr6 cells and the cell cultures of normal fibroblasts in the responsiveness towards WNT5A remain to be elucidated.

Taken together, the in vitro results provide a basis for the understanding of the mechanisms, by which WNT5A exerts its tumor promoting effects also in vivo.

Gene list A: Genes commonly differentiating both tumors from the reference fibrous tissue or solely differentiating one tumor from the reference fibrous tissue

Aggr, Super > Ref
Aggr, Super < Ref
Aggr ≠ Ref or Super ≠ Ref

Genes whose differential expression between fibromatoses and reference tissue has been described in the literature are marked with the according informations in the first column. Abbreviations:

A	**Aggressive** fibromatosis
S	**Superficial** fibromatosis
R	**Reference** fibrous tissue
√	Differential expression described in the **literature** could be **confirmed** by own results
no √	Differential expression described in the **literature could not be confirmed**, the result published in the literature is indicated, e.g. A = R
IHC.	Result published in the literature obtained by **immunohistochemical** analysis
M	Result published in the literature obtained by **microarray** gene expression analysis
W	Result published in the literature obtained by **Western blot** analysis
N	Result published in the literature obtained by **Northern blot** analysis
ELISA	Result published in the literature obtained by **ELISA** analysis
2D-GE	Result published in the literature obtained by **two-dimensional gel electrophoresis**, followed by mass spectrometry (MS) analysis
PCR	Result published in the literature obtained by **RT-PCR** or **real-time RT-PCR**

1) Wnt signalling pathway

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	APC	adenomatosis polyposis coli (APC), mRNA [NM_0000	2.16	1.20E-07	802	377				
	AXIN2	axin 2 (conductin, axil) (AXIN2), mRNA [NM_004655]	2.32	0.00002	711	312	2.18	0.00006	1179	564
			5.54	5.43E-33	3650	661				
A√ IHC,M	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1) (CCN	3.06	1.22E-37	2463	802	2.41	2.61E-23	2102	867
A√ M	CCND2	cyclin D2 (CCND2), mRNA [NM_001759]	2.10	6.80E-12	1014	479	2.72	1.20E-11	913	346
	CCND2		5.16	5.81E-31	1799	350				
	CREBBP	CREB binding protein (Rubinstein-Taybi syndrome) (C	0.49	2.94E-10	405	830				
A√ IHC.	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa (	2.74	2.21E-21	3650	1324				
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	0.13	0	628	5007	0.26	0	1125	4354
	EGFR	epidermal growth factor receptor (erythroblastic leuke	0.41	0.00052	257	658				
A√ M	EPHB3	EPH receptor B3 (EPHB3), mRNA [NM_004443]	3.91	1.60E-14	1113	289	2.87	4.10E-13	857	291
S√ IHC,ISH,W,M	FN1	fibronectin 1 (FN1), transcript variant 7, mRNA [NM_0	2.14	7.8E-09	1306	617	5.86	0	9319	1582
	FN1		6.26	0	101623	16237	5.97	0	92627	15302
	FOSL1	FOS-like antigen 1 (FOSL1), mRNA [NM_005438]	2.13	7.5E-10	1587	736				
	FRAT1	frequently rearranged in advanced T-cell lymphomas (	3.54	4.48E-36	1557	441	2.58	1.14E-13	955	378
	FZD1	frizzled homolog 1 (Drosophila) (FZD1), mRNA [NM_0	3.65	1.90E-27	15258	4131	2.42	7.85E-16	9305	3796
	FZD10	frizzled homolog 10 (Drosophila) (FZD10), mRNA [NM	2.10	4.41E-10	2552	1196				
	FZD4	frizzled homolog 4 (Drosophila) (FZD4), mRNA [NM_0	0.39	0	2230	5650				
	FZD5	frizzled homolog 5 (Drosophila) (FZD5), mRNA [NM_0	0.46	6.92E-16	573	1260				
	GJA1	gap junction protein, alpha 1, 43kDa (connexin 43) (G	14.32	0	51266	3499	4.75	6.31E-08	1048	239
	GJA1		7.27	0	1444	200	6.75	0	25739	3798
	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2), mRNA [NM_00					0.28	0	3698	13227
	JUN	v-jun sarcoma virus 17 oncogene homolog (avian) (JUN), mRNA [NM_002228]					0.46	0	19284	42331
A=R PCR	LEF1	lymphoid enhancer-binding factor 1 (LEF1), mRNA [N	7.67	0	3569	467	3.11	1.69E-13	1260	392
S√ IHC,ELISA,M	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelat	2.05	9.59E-09	5162	2507	3.31	2.63E-31	7670	2317
A=R PCR										
A>R M,PCR	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatina	0.01	0	3050	286788	0.01	0	3432	279991

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
S>R IHC.	MYC	v-myc myelocytomatosis viral oncogene homolog (avi	0.23	0	901	3971	0.29	1.77E-21	1390	4801
	PTTG1	pituitary tumor-transforming 1 (PTTG1), mRNA [NM_0	6.60	3.18E-37	25146	3703	4.53	1.38E-19	14803	3078
	RHOU	ras homolog gene family, member U (RHOU), mRNA	0.30	4.59E-13	216	703	0.35	2.59E-09	244	672
	RUNX2	runt-related transcription factor 2 (RUNX2), transcript	5.72	0	3228	562	3.91	0	1565	398
	SFRP2	secreted frizzled-related protein 2 (SFRP2), mRNA [N	10.58	0	26378	2490	3.66	0	1964	537
			10.13	0	7482	740	2.91	0	3466	1192
			5.73	0	7945	1388	5.98	0	11575	1934
	SFRP4	secreted frizzled-related protein 4 (SFRP4), mRNA [N	16.57	0	29554	1781	30.97	0	73816	2390
	SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	0.32	0	695	2149	0.41	1.24E-20	927	2249
	SMAD3		0.28	1.33E-29	1180	4180	0.47	7.25E-26	2007	4272
	SOX17	SRY (sex determining region Y)-box 17 (SOX17), mRI	2.03	9.57E-20	2761	1360	2.09	4.30E-29	3075	1473
	TCF4	transcription factor 4 (TCF4), mRNA [NM_003199]	2.23	2.64E-36	14206	6354				
	TNFRSF19	tumor necrosis factor receptor superfamily, member 1	20.61	3.90E-28	3316	158	6.43	2.25E-16	866	127
	TNFRSF19	tumor necrosis factor receptor superfamily, member 1	2.38	0.0823	178	77				
	TWIST1	twist homolog 1 (acrocephalosyndactyly 3; Saethre-Cl	3.54	0	2351	664				
A√ M	WISP1	WNT1 inducible signaling pathway protein 1 (WISP1),	3.03	0	9133	3017	2.57	0.00001	4174	1751
A√ M	WNT5A	wingless-type MMTV integration site family, member 5	14.40	0	5461	378	2.27	4.47E-09	657	295
	WNT6	wingless-type MMTV integration site family, member 6	2.54	6.10E-19	17815	6961				
	WNT11	wingless-type MMTV integration site family, member 1	0.43	2.61E-37	1585	3710	0.40	0	1249	3114

2) TGFβ signalling pathway

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ACVR1B	activin A receptor, type IB (ACVR1B), transcript varian	2.23	4.12E-37	44664	19989				
	AMH	anti-Mullerian hormone (AMH), mRNA [NM_000479]	2.11	3.39E-07	870	407				
A√ M	ASPN	asporin (LRR class 1) (ASPN), mRNA [NM_017680]	14.89	0	36546	2424	27.27	0	91077	3308
S√ PCR	BMP1	bone morphogenetic protein 1 (BMP1), transcript varia	2.04	3.6E-07	720	355				
			2.01	4.1E-08	626	310				
S=R PCR	BMP7	bone morphogenetic protein 7 (osteogenic protein 1) (	16.33	0	2205	136	5.36	9.92E-13	552	99
	BMP8A	bone morphogenetic protein 8a (BMP8A), mRNA [NM	2.90	8.89E-17	47854	16023	2.31	3.31E-12	36604	15440
	CILP	cartilage intermediate layer protein, nucleotide pyroph	0.04	0	3778	87300	0.17	2.75E-22	12084	74050
	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDK	0.26	2.80E-45	863	3382	0.31	1.77E-39	986	3235
S√ IHC,W	COL1A1	collagen, type I, alpha 1 (COL1A1), mRNA [NM_0000	12.42	4.8E-44	434853	32173	13.40	0	377400	28676
A√ PCR,M										
S√ IHC,W	COL1A2	collagen, type I, alpha 2 (COL1A2), mRNA [NM_0000	8.36	1.1E-44	3978	468	25.72	0	360611	14122
	COL1A2		21.97	1.4E-45	438794	18042	5.50	0	2176	396
SvR √	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome ty	15.12	0	338160	21395	22.10	0	418071	18917
AvR √ M,PCR			19.81	0	439519	20690	33.25	0	66275	1999
			40.43	0	117501	2996	13.65	0	329376	24085
	CTHRC1	collagen triple helix repeat containing 1 (CTHRC1), m	13.13	0	62754	4696	7.61	0	40183	5219
S√ M	DCN	decorin (DCN), transcript variant A1, mRNA [NM_001	0.39	1.34E-11	60964	157385	0.40	1.90E-15	54925	139728
S√ IHC,ISH,W,M	FN1	fibronectin 1 (FN1), transcript variant 7, mRNA [NM_0	2.14	7.8E-09	1306	617	5.86	0	9319	1582
	FN1		6.26	0	101623	16237	5.97	0	92627	15302
	GADD45B	growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA [NM_015675]					0.34	1.29E-23	10475	31156
	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2), mRNA [NM_00					0.28	0	3698	13227
	INHBB	inhibin, beta B (activin AB beta polypeptide) (INHBB),	0.39	6.61E-31	1704	4376	0.30	3.39E-27	1254	4220
S√ IHC,ELISA,M	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelat	2.05	9.59E-09	5162	2507	3.31	2.63E-31	7670	2317
A=R PCR										
S>R IHC.	MYC	v-myc myelocytomatosis viral oncogene homolog (avi	0.23	0	901	3971	0.29	1.77E-21	1390	4801
	NODAL	nodal homolog (mouse) (NODAL), mRNA [NM_01805	2.23	7.82E-07	1184	539	2.14	4.05E-13	871	413
	OGN	osteoglycin (osteoinductive factor, mimecan) (OGN), t	16.59	0	8686	524	7.88	0	3775	480
	SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	0.32	0	695	2149	0.41	1.24E-20	927	2249
	SMAD3		0.28	1.33E-29	1180	4180	0.47	7.25E-26	2007	4272
S√ M	SPARC	secreted protein, acidic, cysteine-rich (osteonectin) (S	12.85	1.1E-44	440326	31435	13.33	0	433979	31064

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
A√ M	TGFB3	transforming growth factor, beta 3 (TGFB3), mRNA [N	9.31	0	6452	684	8.77	0	6492	736
S√ IHC.	TGFB3						5.77	0.0002	305	64
S√ 2D-GE	TGFBI	transforming growth factor, beta-induced, 68kDa (TGF	4.56	1.2E-33	162242	35015	7.27	0	289821	39235
S√ IHC, ELISA,M	TGFB2	transforming growth factor, beta 2 (TGFB2), mRNA [NM_003238]					3.06	1.82E-11	703	238
A>R W	TGFBR2	transforming growth factor, beta receptor II (70/80kDa	0.44	4.88E-15	382	861				
A=R W	TGFBR3	transforming growth factor, beta receptor III (betaglyca	0.35	2.62E-32	744	2148	0.27	0	558	2087
	THBS2	thrombospondin 2 (THBS2), mRNA [NM_003247]	6.57	1.21E-33	18698	2782	5.36	0	12664	2355
	TINP1	TGF beta-inducible nuclear protein 1 (TINP1), mRNA	0.46	2.38E-18	5486	12150	0.48	9.30E-20	4510	9552

3) PI3K-AKT signalling pathway

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
A√ IHC,M	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1) (CCN	3.06	1.22E-37	2463	802	2.41	2.61E-23	2102	867
	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDK	0.26	2.80E-45	863	3382	0.31	1.77E-39	986	3235
A√ IHC.	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa (	2.74	2.21E-21	3650	1324				
	EGFR	epidermal growth factor receptor (erythroblastic leuke	0.41	0.00052	257	658				
	FGF1	fibroblast growth factor 1 (acidic) (FGF1), transcript va	4.24	0	1627	384	3.49	1.67E-27	1720	495
	FGF7	fibroblast growth factor 7 (keratinocyte growth factor) (	0.46	1.54E-27	766	1683	0.32	1.40E-45	510	1616
	FGF7	fibroblast growth factor 7 (keratinocyte growth factor) (	0.29	2.64E-22	3824	13118	0.19	0	2448	12905
	FOXO1A	forkhead box O1A (rhabdomyosarcoma) (FOXO1A), n	0.28	0	3545	12921	0.28	0	3417	12156
	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-l	2.27	1.37E-43	9240	4072				
A √ M,W,N	IGFBP6	insulin-like growth factor binding protein 6 (IGFBP6), r	0.09	0	380	4180	0.25	6.62E-35	1172	4684
	IL6R	interleukin 6 receptor (IL6R), transcript variant 1, mRN	0.27	8.10E-26	401	1497	0.25	4.75E-30	391	1540
	IL20RA	interleukin 20 receptor, alpha (IL20RA), mRNA [NM_0	2.66	7.34E-11	1032	381				
	IRS1	insulin receptor substrate 1 (IRS1), mRNA [NM_00554	3.33	0	2352	706	2.85	2.80E-45	1796	632
	IRS2	insulin receptor substrate 2 (IRS2), mRNA [NM_00374	0.29	0	656	2298	0.25	0	574	2252
S>R IHC.	MYC	v-myc myelocytomatosis viral oncogene homolog (avi	0.23	0	901	3971	0.29	1.77E-21	1390	4801
	NFKBIA	nuclear factor of kappa light polypeptide gene enhanc	0.37	0	11092	29643	0.25	0	8082	32193
	PDGFC	platelet derived growth factor C (PDGFC), mRNA [NM	2.19	6.40E-20	3513	1597	2.44	0	3722	1522
	PDK1	pyruvate dehydrogenase kinase, isoenzyme 1 (PDK1)	0.45	3.84E-11	557	1224	0.45	2.21E-26	663	1459
A=S√ IHC,PCR	TP53	tumor protein p53 (Li-Fraumeni syndrome) (TP53), mR	0.21	0	614	2968	0.27	0	726	2711
S>R W										
	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide	4.87	0	5856	1202	4.62	0	5517	1192
	PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma) (PIK3R3), mRNA [NM_0036					0.48	8.45E-06	322	707
	VEGF	vascular endothelial growth factor, mRNA (cDNA clone MGC:70609 IMAGE:6006890), com					0.48	5.79E-16	1188	2446

4) Extracellular matrix (ECM)

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
A√ M	ADAM12	a disintegrin and metalloproteinase domain 12 (meltri	3.52	7.16E-40	6058	1722	3.05	0	5308	1737
S√ PCR										
	ADAMTSL3	ADAMTS-like 3 (ADAMTSL3), mRNA [NM_207517]	0.11	9.31E-07	67	574	0.18	1.59E-12	120	615
	ADAMTS5	a disintegrin-like and metalloprotease (reprolysin type	0.19	0	2709	14432	0.34	7.66E-20	5514	16061
	AGRN	agrin (AGRN), mRNA [NM_198576]	2.00	1.58E-32	5920	2949				
	ANGPTL7	angiopoietin-like 7 (ANGPTL7), mRNA [NM_021146]	0.005	0	1284	274406	0.05	0	11741	233940
S√ M	APP	amyloid beta (A4) precursor protein (protease nexin-II	4.67	1.64E-27	1801	387	3.80	2.09E-22	1778	472
A√ M	ASPN	asporin (LRR class 1) (ASPN), mRNA [NM_017680]	14.89	0	36546	2424	27.27	0	91077	3308
	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39) (CHI3L1),	0.10	0	550	5400	0.14	0	711	4990
	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39) (CHI3L1),	0.18	5.72E-14	152	829				
	CHI3L2	chitinase 3-like 2 (CHI3L2), mRNA [NM_004000]	0.09	0	362	4237	0.13	0	485	3719
	CILP	cartilage intermediate layer protein, nucleotide pyroph	0.04	0	3778	87300	0.17	2.75E-22	12084	74050
	COL10A1	collagen, type X, alpha 1(Schmid metaphyseal chondr	2.61	6.42E-39	5005	1922				
A√ M	COL11A1	collagen, type XI, alpha 1 (COL11A1), transcript varia	16.45	0	11610	708	5.43	0	3027	556
	COL11A2	collagen, type XI, alpha 2 (COL11A2), transcript varia	0.55	3.6E-13	2073	3787				
A√ M	COL12A1	collagen, type XII, alpha 1 (COL12A1), transcript varia	2.32	0	5029	2168				
	COL13A1	collagen, type XIII, alpha 1 (COL13A1), transcript varia	7.79	0	3009	383	9.53	0	3369	351
A√ M	COL14A1	collagen, type XIV, alpha 1 (undulin) (COL14A1), mRN	5.93	0	9815	1652	6.47	0	11169	1727
	COL14A1		8.62	0	15418	1794	12.96	0	28779	2173
	COL14A1		2.83	0	12313	4344				
	COL16A1	collagen, type XVI, alpha 1 (COL16A1), mRNA [NM_0	9.20	1.29E-29	151140	15768	5.84	2.36E-22	84214	13909
	COL18A1	collagen, type XVIII, alpha 1 (COL18A1), transcript va	2.57	0	31927	12408	3.17	0	38523	12135
S√ IHC,W	COL1A1	collagen, type I, alpha 1 (COL1A1), mRNA [NM_0000	12.42	4.8E-44	434853	32173	13.40	0	377400	28676
A√ PCR,M										
S√ IHC,W	COL1A2	collagen, type I, alpha 2 (COL1A2), mRNA [NM_0000	8.36	1.1E-44	3978	468	25.72	0	360611	14122
	COL1A2		21.97	1.4E-45	438794	18042	5.50	0	2176	396
	COL25A1	collagen, type XXV, alpha 1 (COL25A1), transcript var	2.20	1.15E-17	1223	558				
SvR √	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome ty	15.12	0	338160	21395	22.10	0	418071	18917
AvR √ M,PCR			19.81	0	439519	20690	33.25	0	66275	1999
			40.43	0	117501	2996	13.65	0	329376	24085
S√ IHC.	COL4A1	collagen, type IV, alpha 1 (COL4A1), mRNA [NM_001	2.87	1.20E-12	762	263	2.05	0.00081	596	268
S√ IHC.	COL4A5	collagen, type IV, alpha 5 (Alport syndrome) (COL4A5	2.30	3.38E-13	805	351	4.00	0	2598	649
			2.32	9.87E-21	1315	564				
			7.93	0	7045	883				
A√ M	COL5A1	collagen, type V, alpha 1 (COL5A1), mRNA [NM_0000	12.82	0	53506	4187	7.85	0	23475	3005
			28.83	0	5889	206	15.60	0	2091	134
A,S√ M	COL5A2	collagen, type V, alpha 2 (COL5A2), mRNA [NM_0003	18.18	0	18974	1044	16.16	0	20278	1262
	COL5A2		6.34	0	73717	11539	5.79	0	69785	11871
	COL5A2		4.17	4.40E-43	1473	354	4.08	5.96E-38	1443	357
	COL5A3	collagen, type V, alpha 3 (COL5A3), mRNA [NM_0157	2.12	6.86E-12	816	386				
S√ IHC.	COL6A1	collagen, type VI, alpha 1 (COL6A1), mRNA [NM_001	4.13	0	2438	588	7.52	0	366939	48166
A√ M	COL6A1		7.50	4.68E-29	384091	47253	3.34	3.63E-34	1405	425
S√ IHC.	COL6A2	collagen, type VI, alpha 2 (COL6A2), transcript variant	10.68	0	4702	440	9.25	0	3564	384
A√ M	COL6A2		2.55	3.22E-18	3551	1388	2.03	1.22E-13	2717	1334
S√ IHC,2D-GE	COL6A3	collagen, type VI, alpha 3 (COL6A3), transcript variant	2.22	0	76955	34656	2.71	1.17E-17	94430	34605
A√ M										
S√ M	COL8A1	collagen, type VIII, alpha 1 (COL8A1), transcript varia	3.10	7.17E-23	8420	2674	2.47	0	5304	2144
	COL8A2	collagen, type VIII, alpha 2 (COL8A2), mRNA [NM_00	2.20	5.66E-15	4848	2176	5.85	0	14811	2511
	CTHRC1	collagen triple helix repeat containing 1 (CTHRC1), m	13.13	0	62754	4696	7.61	0	40183	5219
S√ M	DCN	decorin (DCN), transcript variant A1, mRNA [NM_001	0.39	1.34E-11	60964	157385	0.40	1.90E-15	54925	139728
	ECM1	extracellular matrix protein 1 (ECM1), transcript varian	4.14	0	2572	621	4.59	0	3023	658
	EFEMP2	EGF-containing fibulin-like extracellular matrix protein	2.52	0	3065	1215				
A,S√ M	FAP	fibroblast activation protein, alpha (FAP), mRNA [NM_	52.70	0	56300	1063	40.41	0	41456	1023

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	FBN1	fibrillin 1 (Marfan syndrome) (FBN1), mRNA [NM_000	3.72	0	16665	4470	2.53	9.42E-43	10616	4188
	FIBL-6	hemicentin (FIBL-6), mRNA [NM_031935]	4.52	0	5522	1221	2.85	3.12E-10	2444	876
S√ IHC,ISH,W,M	FN1	fibronectin 1 (FN1), transcript variant 7, mRNA [NM_0	2.14	7.8E-09	1306	617	5.86	0	9319	1582
	FN1		6.26	0	101623	16237	5.97	0	92627	15302
	FN1		6.90	0	14416	2083				
	LAMA1	laminin, alpha 1 (LAMA1), mRNA [NM_005559]	2.21	4.92E-08	626	281	2.36	4.13E-07	554	228
	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystro	0.46	0	3377	7359	0.46	4.12E-31	3706	8078
	LAMA4	laminin, alpha 4 (LAMA4), mRNA [NM_002290]	3.81	1.56E-23	14402	3748	3.17	4.53E-23	10314	3223
S√ IHC.	LAMB1	laminin, beta 1 (LAMB1), mRNA [NM_002291]	3.56	0	25964	7281	2.85	0	19914	6971
S√ 2D-GE	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1) (LG/	4.86	0	37051	7543	5.12	0	39357	7656
	LUM	lumican (LUM), mRNA [NM_002345]	6.17	0	82530	13378	3.76	1.21E-30	43130	11393
	MFAP5	microfibrillar associated protein 5 (MFAP5), mRNA [N	4.90	3.39E-40	6723	1352				
	MGP	matrix Gla protein (MGP), mRNA [NM_000900]	0.37	5.92E-30	29059	78907				
S√ IHC,ELISA,M	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelat	2.05	9.59E-09	5162	2507	3.31	2.63E-31	7670	2317
A=R PCR										
	MMP10	matrix metalloproteinase 10 (stromelysin 2) (MMP10),	0.29	0	1013	3514	0.24	0	832	3401
A√ PCR	MMP11	matrix metalloproteinase 11 (stromelysin 3) (MMP11),	18.57	0	21077	1139	8.93	0	6272	693
A,S√ PCR	MMP14	matrix metalloproteinase 14 (membrane-inserted) (MMP14), mRNA [NM_004995]					2.29	1.56E-15	4216	1826
	MMP19	matrix metalloproteinase 19 (MMP19), transcript varia	10.78	0	3898	362	3.43	4.58E-21	1259	360
	MMP23B	matrix metalloproteinase 23B (MMP23B), mRNA [NM_	6.09	0	4386	719	2.75	1.54E-42	1210	439
	MMP27	matrix metalloproteinase 27 (MMP27), mRNA [NM_02	2.07	1.87E-17	1608	774				
A>R M,PCR	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatina	0.01	0	3050	286788	0.01	0	3432	279991
A<R M	NID2	nidogen 2 (osteonidogen) (NID2), mRNA [NM_007361	7.30	0	2909	396	12.24	0	5659	460
	OGN	osteoglycin (osteoinductive factor, mimecan) (OGN), t	16.59	0	8686	524	7.88	0	3775	480
	P4HA2	procollagen-proline, 2-oxoglutarate 4-dioxygenase (pr	12.03	0	25382	2106	7.78	0	15322	1960
	P4HA3	procollagen-proline, 2-oxoglutarate 4-dioxygenase (pr	3.04	1.21E-18	975	323	10.32	0	6331	612
	P4HA3		7.21	0	3635	504	4.29	5.01E-29	1302	309
	P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (pr	3.61	0	11836	3279	2.70	0	7759	2870
	P4HB						2.41	5.50E-09	49702	20517
	PCOLCE	procollagen C-endopeptidase enhancer (PCOLCE), m	5.17	0	11955	2298	3.13	0	6771	2171
	PCOLCE2	procollagen C-endopeptidase enhancer 2 (PCOLCE2)	0.03	0	1250	46252	0.05	0	2015	44588
	PLG	plasminogen (PLG), mRNA [NM_000301]	5.56	0	43311	7793	3.39	0	24010	7092
	PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	2.29	7.21E-27	6001	2614	2.39	1.53E-20	6111	2543
S√ IHC,M,PCR	POSTN	periostin, osteoblast specific factor (POSTN), mRNA [	53.68	0	36596	691	60.23	0	85449	1436
	PRG2	proteoglycan 2, bone marrow (natural killer cell activat	2.28	2.54E-18	4001	1749				
S√ M	PRG4	proteoglycan 4 (PRG4), mRNA [NM_005807]	0.01	0	482	44080	0.12	0	4826	40787
	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A (alpha	0.08	0	526	6291	0.09	0	544	5783
	SERPINA3		0.15	0	426	2793	0.21	0	477	2342
	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha	4.73	4.20E-16	3499	728	2.80	9.34E-08	1838	668
	SERPINA1(	serine (or cysteine) proteinase inhibitor, clade A (alpha	2.59	3.16E-31	1487	575				
	SERPING1	serine (or cysteine) proteinase inhibitor, clade G (C1 in	0.26	0	1289	4950	0.34	3.82E-39	1445	4214
S√ M,W	SERPINH1	serine (or cysteine) proteinase inhibitor, clade H (heat	9.87	0	4017	406	5.35	2.61E-38	2103	395
	SMOC2	SPARC related modular calcium binding 2 (SMOC2),	3.45	8.2E-13	12678	3473	3.71	3.23E-15	13922	3652
S√ M	SPARC	secreted protein, acidic, cysteine-rich (osteonectin) (S	12.85	1.1E-44	440326	31435	13.33	0	433979	31064
	SPON1	spondin 1, extracellular matrix protein (SPON1), mRN	35.89	0	41573	1161	18.04	0	18125	1001
A√ M	SPON2	spondin 2, extracellular matrix protein (SPON2), mRN	9.59	1.19E-41	103308	10375	23.98	0	278300	11395
	THBS2	thrombospondin 2 (THBS2), mRNA [NM_003247]	6.57	1.21E-33	18698	2782	5.36	0	12664	2355
S√ IHC.	TIMP2	tissue inhibitor of metalloproteinase 2 (TIMP2), mRNA	2.27	1.28E-08	6918	3005	2.04	1.74E-10	6273	3038
A=R PCR										
A√ PCR	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus	0.46	9.58E-23	4559	9945				
	TIMP4	tissue inhibitor of metalloproteinase 4 (TIMP4), mRNA	0.35	0	1165	3367	0.31	2.46E-32	916	2958
S√ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_00216(	2.48	0	5955	2403	8.99	0	41901	4677

5) ECM-receptor interaction

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	AGRN	agrin (AGRN), mRNA [NM_198576]	2.00	1.58E-32	5920	2949				
	ACTB	actin, beta (ACTB), mRNA [NM_001101]	2.11	2.8E-19	35553	16645	2.01	0	53091	26364
	ACTN1	actinin, alpha 1 (ACTN1), mRNA [NM_001102]	2.20	1.46E-15	3378	1532	2.14	2.18E-26	3488	1626
	ACTN3	actinin, alpha 3 (ACTN3), mRNA [NM_001104]	2.29	3.01E-06	701	312	2.05	0.00036	531	274
	BIRC3	baculoviral IAP repeat-containing 3 (BIRC3), transcrip	0.20	1.31E-33	238	1208	0.22	4.44E-35	269	1236
	BIRC7	baculoviral IAP repeat-containing 7 (livin) (BIRC7), tra	0.36	1.48E-08	504	1430	0.35	2.14E-15	504	1447
	CAPN1	calpain 1, (mu/I) large subunit (CAPN1), mRNA [NM_(	2.19	4.97E-08	635	291				
	CAPN5	calpain 5 (CAPN5), mRNA [NM_004055]	2.95	0	4144	1406				
	CAPN6	calpain 6 (CAPN6), mRNA [NM_014289]	3.97	1.05E-32	2581	651	4.95	4.42E-30	2975	599
	CAST	calpastatin (CAST), transcript variant 2, mRNA [NM_1	0.41	3.27E-07	5187	12975	0.49	2.15E-06	6167	12760
	CAV1	caveolin 1, caveolae protein, 22kDa (CAV1), mRNA [N	0.38	0	3179	8358				
	CAV2	caveolin 2 (CAV2), transcript variant 1, mRNA [NM_0(	0.49	1.14E-06	401	823				
	CD36	CD36 antigen (collagen type I receptor, thrombospond	0.29	4.39E-37	722	2495	0.27	0	584	2225
	CLK1	CDC-like kinase 1 (CLK1), mRNA [NM_004071]	0.29	0	1087	3737	0.30	0	1107	3715
	CLK1		0.32	8.92E-22	2024	6304	0.35	5.48E-21	2077	6045
	COL10A1	collagen, type X, alpha 1(Schmid metaphyseal chondr	2.61	6.42E-39	5005	1922				
A√ M	COL11A1	collagen, type XI, alpha 1 (COL11A1), transcript varia	16.45	0	11610	708	5.43	0	3027	556
	COL11A2	collagen, type XI, alpha 2 (COL11A2), transcript varia	0.55	3.6E-13	2073	3787				
A√ M	COL12A1	collagen, type XII, alpha 1 (COL12A1), transcript varia	2.32	0	5029	2168				
	COL13A1	collagen, type XIII, alpha 1 (COL13A1), transcript varia	7.79	0	3009	383	9.53	0	3369	351
A√ M	COL14A1	collagen, type XIV, alpha 1 (undulin) (COL14A1), mRN	5.93	0	9815	1652	6.47	0	11169	1727
	COL14A1		8.62	0	15418	1794	12.96	0	28779	2173
	COL14A1		2.83	0	12313	4344				
	COL16A1	collagen, type XVI, alpha 1 (COL16A1), mRNA [NM_0	9.20	1.29E-29	151140	15768	5.84	2.36E-22	84214	13909
	COL18A1	collagen, type XVIII, alpha 1 (COL18A1), transcript va	2.57	0	31927	12408	3.17	0	38523	12135
S√ IHC,W	COL1A1	collagen, type I, alpha 1 (COL1A1), mRNA [NM_0000	12.42	4.8E-44	434853	32173	13.40	0	377400	28676
A√ PCR,M										
S√ IHC,W	COL1A2	collagen, type I, alpha 2 (COL1A2), mRNA [NM_0000	8.36	1.1E-44	3978	468	25.72	0	360611	14122
	COL1A2		21.97	1.4E-45	438794	18042	5.50	0	2176	396
	COL25A1	collagen, type XXV, alpha 1 (COL25A1), transcript var	2.20	1.15E-17	1223	558				
S√	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome ty	15.12	0	338160	21395	22.10	0	418071	18917
A√ M,PCR			19.81	0	439519	20690	33.25	0	66275	1999
			40.43	0	117501	2996	13.65	0	329376	24085
S√ IHC.	COL4A1	collagen, type IV, alpha 1 (COL4A1), mRNA [NM_001	2.87	1.20E-12	762	263	2.05	0.00081	596	268
S√ IHC.	COL4A5	collagen, type IV, alpha 5 (Alport syndrome) (COL4A5	2.30	3.38E-13	805	351	4.00	0	2598	649
			2.32	9.87E-21	1315	564				
			7.93	0	7045	883				
A√ M	COL5A1	collagen, type V, alpha 1 (COL5A1), mRNA [NM_0000	12.82	0	53506	4187	7.85	0	23475	3005
			28.83	0	5889	206	15.60	0	2091	134
A,S√ M	COL5A2	collagen, type V, alpha 2 (COL5A2), mRNA [NM_0003	18.18	0	18974	1044	16.16	0	20278	1262
	COL5A2		6.34	0	73717	11539	5.79	0	69785	11871
	COL5A2		4.17	4.40E-43	1473	354	4.08	5.96E-38	1443	357
	COL5A3	collagen, type V, alpha 3 (COL5A3), mRNA [NM_0157	2.12	6.86E-12	816	386				
S√ IHC.	COL6A1	collagen, type VI, alpha 1 (COL6A1), mRNA [NM_001	4.13	0	2438	588	7.52	0	366939	48166
A√ M	COL6A1		7.50	4.68E-29	384091	47253	3.34	3.63E-34	1405	425
S√ IHC.	COL6A2	collagen, type VI, alpha 2 (COL6A2), transcript variant	10.68	0	4702	440	9.25	0	3564	384
A√ M	COL6A2		2.55	3.22E-18	3551	1388	2.03	1.22E-13	2717	1334
S√ IHC,2D-GE	COL6A3	collagen, type VI, alpha 3 (COL6A3), transcript variant	2.22	0	76955	34656	2.71	1.17E-17	94430	34605
A√ M										
S√ M	COL8A1	collagen, type VIII, alpha 1 (COL8A1), transcript varia	3.10	7.17E-23	8420	2674	2.47	0	5304	2144
	COL8A2	collagen, type VIII, alpha 2 (COL8A2), mRNA [NM_00	2.20	5.66E-15	4848	2176	5.85	0	14811	2511
	CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-	10.01	0	2999	300	6.22	0	1946	311
A√ IHC.	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa (	2.74	2.21E-21	3650	1324				

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	DIAPH1	diaphanous homolog 1 (Drosophila) (DIAPH1), mRNA	0.31	4.57E-20	8246	27359				
	DIAPH3	diaphanous homolog 3 (Drosophila) (DIAPH3), mRNA	4.72	3.32E-34	1884	401	3.05	8.92E-18	1248	416
	DYRK2	dual-specificity tyrosine-(Y)-phosphorylation regulated	3.12	0	1863	596	2.61	7.19E-20	1513	571
	EDIL3	EGF-like repeats and discoidin I-like domains 3 (EDIL	8.77	0	8573	968	20.14	0	18664	922
	EGFR	epidermal growth factor receptor (erythroblastic leuke	0.41	0.00052	257	658				
	FLNC	filamin C, gamma (actin binding protein 280) (FLNC),	3.04	0.00033	752	260	2.76	0.00018	681	277
	FLRT2	fibronectin leucine rich transmembrane protein 2 (FLR	4.42	1.83E-10	709	156	4.22	3.18E-19	981	240
	FLRT2		3.04	1.22E-14	807	268	6.29	0	1540	244
S√ IHC,ISH,W,M	FN1	fibronectin 1 (FN1), transcript variant 7, mRNA [NM_0	2.14	7.8E-09	1306	617	5.86	0	9319	1582
	FN1		6.26	0	101623	16237	5.97	0	92627	15302
	FN1		6.90	0	14416	2083				
	GPC1	glypican 1 (GPC1), mRNA [NM_002081]	3.40	0	34796	10218				
	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypepti	3.14	6.26E-22	1077	344	4.90	8.06E-32	1832	378
			2.94	0	10769	3662	5.04	0	19513	3872
S√ IHC, M	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide	2.59	6.32E-24	5992	2307	3.98	3.05E-32	9727	2446
	ITGB1		2.64	0	18176	6883	3.61	0	25574	7102
	ITGB1		2.82	5.61E-13	53401	18303	4.51	2.48E-23	92904	19961
	ITGB5	integrin, beta 5 (ITGB5), mRNA [NM_002213]	3.10	1.38E-17	7018	2197				
	JUN	v-jun sarcoma virus 17 oncogene homolog (avian) (JUN), mRNA [NM_002228]					0.46	0	19284	42331
	LAMA1	laminin, alpha 1 (LAMA1), mRNA [NM_005559]	2.21	4.92E-08	626	281	2.36	4.13E-07	554	228
	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystro	0.46	0	3377	7359	0.46	4.12E-31	3706	8078
	LAMA4	laminin, alpha 4 (LAMA4), mRNA [NM_002290]	3.81	1.56E-23	14402	3748	3.17	4.53E-23	10314	3223
S√ IHC.	LAMB1	laminin, beta 1 (LAMB1), mRNA [NM_002291]	3.56	0	25964	7281	2.85	0	19914	6971
	MFAP5	microfibrillar associated protein 5 (MFAP5), mRNA [N	4.90	3.39E-40	6723	1352				
A<R M	NID2	nidogen 2 (osteonidogen) (NID2), mRNA [NM_00736	7.30	0	2909	396	12.24	0	5659	460
	NLGN1	neuroligin 1 (NLGN1), mRNA [NM_014932]	0.15	3.13E-14	123	791	0.21	1.23E-43	235	1118
	PDGFC	platelet derived growth factor C (PDGFC), mRNA [NM	2.19	6.40E-20	3513	1597	2.44	0	3722	1522
	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide	4.87	0	5856	1202	4.62	0	5517	1192
S√ IHC,M,PCR	POSTN	periostin, osteoblast specific factor (POSTN), mRNA [	53.68	0	36596	691	60.23	0	85449	1436
	PPP1CB	protein phosphatase 1, catalytic subunit, beta isoform	2.16	2.92E-07	2033	935				
	RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1 (RA	4.70	0	13411	2853	2.78	7.29E-44	7895	2853
	SDC1	syndecan 1 (SDC1), transcript variant 1, mRNA [NM_	9.81	0	6976	700	4.68	3.17E-24	2507	533
	SDC4	syndecan 4 (amphiglycan, ryudocan) (SDC4), mRNA	3.38	1.59E-42	108824	32179	2.11	1.40E-45	57062	27002
	SGCE	sarcoglycan, epsilon (SGCE), mRNA [NM_003919]	3.33	0	22127	6649				
	SHC1	SHC (Src homology 2 domain containing) transformin	2.15	4.28E-24	3620	1681				
A√ M	SPON2	spondin 2, extracellular matrix protein (SPON2), mRN	9.59	1.19E-41	103308	10375	23.98	0	278300	11395
	SV2A	synaptic vesicle glycoprotein 2A (SV2A), mRNA [NM_	3.05	0	62284	20411	2.36	0	37972	16114
	THBS2	thrombospondin 2 (THBS2), mRNA [NM_003247]	6.57	1.21E-33	18698	2782	5.36	0	12664	2355
S√ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_00216	2.48	0	5955	2403	8.99	0	41901	4677
	VASP	vasodilator-stimulated phosphoprotein (VASP), transc	2.65	1.87E-14	6738	2501	2.29	3.40E-13	5901	2538
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	5.48	0	2197	401	2.21	1.07E-09	649	289

6) Adherens junction and cell adhesion molecules (CAMs)

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	AEBP1	AE binding protein 1 (AEBP1), mRNA [NM_001129]	2.79	6.98E-16	8323	2947	2.85	2.02E-16	8270	2829
	AGRN	agrin (AGRN), mRNA [NM_198576]	2.00	1.58E-32	5920	2949				
S√ M	APP	amyloid beta (A4) precursor protein (protease nexin-II	4.67	1.64E-27	1801	387	3.80	2.09E-22	1778	472
	ASTN2	astrotactin 2 (ASTN2), transcript variant 1, mRNA [NM	2.18	0	3932	1803				
	CASK	calcium/calmodulin-dependent serine protein kinase (	3.37	1.31E-41	1100	326	2.24	2.24E-07	719	309
	CCR1	chemokine (C-C motif) receptor 1 (CCR1), mRNA [NM	0.32	0	1666	5273	0.42	0	1849	4415
	CD226	CD226 antigen (CD226), mRNA [NM_006566]	2.52	1.40E-10	682	269				
	CD36	CD36 antigen (collagen type I receptor, thrombospond	0.29	4.39E-37	722	2495	0.27	0	584	2225
	CDH11	cadherin 11, type 2, OB-cadherin (osteoblast) (CDH11	3.86	7.05E-37	19002	4897	3.21	5.52E-39	16289	5038
	CDH19	cadherin 19, type 2 (CDH19), mRNA [NM_021153]	0.12	2.87E-41	121	1038	0.09	7.42E-42	83	923
	CDH19		0.49	0.55828	38	75				
	CDH24	cadherin-like 24 (CDH24), mRNA [NM_022478]	2.23	2.28E-13	4982	2230				
	CHL1	cell adhesion molecule with homology to L1CAM (clos	3.70	2.78E-12	511	138				
	CHST4	carbohydrate (N-acetylglucosamine 6-O) sulfotransfer	0.42	8.15E-41	1639	3862	0.45	1.28E-27	1917	4238
	CRTAC1	cartilage acidic protein 1 (CRTAC1), mRNA [NM_018	0.07	0	190	2558	0.20	0	539	2753
A√ IHC.	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa (	2.74	2.21E-21	3650	1324				
	CX3CL1	chemokine (C-X3-C motif) ligand 1 (CX3CL1), mRNA	0.32	0	1437	4539	0.25	0	1041	4250
	CXCR3	chemokine (C-X-C motif) receptor 3 (CXCR3), mRNA	2.58	9.20E-32	5252	2029				
	DCBLD2	discoidin, CUB and LCCL domain containing 2 (DCBL	2.12	8.96E-14	3810	1780	2.02	7.98E-14	3823	1879
	DPT	dermatopontin (DPT), mRNA [NM_001937]	9.96	0	2257	226	9.95	0	2923	293
	EDG1	endothelial differentiation, sphingolipid G-protein-coup	0.27	1.06E-11	194	716	0.38	4.81E-08	235	598
	EMILIN1	elastin microfibril interfacer 1 (EMILIN1), mRNA [NM_	2.15	6.22E-26	10251	4755				
	FAT	FAT tumor suppressor homolog 1 (Drosophila) (FAT),	2.89	1.40E-45	4138	1430	3.28	0	4241	1294
	FIBL-6	hemicentin (FIBL-6), mRNA [NM_031935]	4.52	0	5522	1221	2.85	3.12E-10	2444	876
	FLRT2	fibronectin leucine rich transmembrane protein 2 (FLR	4.42	1.83E-10	709	156	4.22	3.18E-19	981	240
	FLRT2		3.04	1.22E-14	807	268	6.29	0	1540	244
	GJA1	gap junction protein, alpha 1, 43kDa (connexin 43) (G	14.32	0	51266	3499	4.75	6.31E-08	1048	239
	GJA1		7.27	0	1444	200	6.75	0	25739	3798
	HNT	neurotrimin (HNT), mRNA [NM_016522]	3.31	7.91E-17	971	296	4.05	1.86E-34	1397	348
	KAL1	Kallmann syndrome 1 sequence (KAL1), mRNA [NM_	10.21	1.53E-26	698	68	6.53	9.23E-14	539	80
A=R PCR	LEF1	lymphoid enhancer-binding factor 1 (LEF1), mRNA [N	7.67	0	3569	467	3.11	1.69E-13	1260	392
	LOXL2	lysyl oxidase-like 2 (LOXL2), mRNA [NM_002318]	3.16	0	22483	7028	3.95	0	28934	7237
	LRRN5	leucine rich repeat neuronal 5 (LRRN5), transcript var	2.62	0	17464	6662				
	LU	Lutheran blood group (Auberger b antigen included) (L	3.03	4.68E-15	33840	10984				
	MMRN1	multimerin 1 (MMRN1), mRNA [NM_007351]	2.49	2.68E-22	1628	657				
	NEO1	neogenin homolog 1 (chicken) (NEO1), mRNA [NM_0	2.52	5.87E-25	2826	1123	2.14	3.75E-33	2427	1136
	NLGN1	neuroligin 1 (NLGN1), mRNA [NM_014932]	0.15	3.13E-14	123	791	0.21	1.23E-43	235	1118
	PCDH7	BH-protocadherin (brain-heart) (PCDH7), transcript va	2.66	4.57E-23	938	353	2.75	2.98E-30	1160	423
							19.74	8.38E-25	740	40
							2.29	0.11056	140	60
	PC-LKC	protocadherin LKC (PC-LKC), mRNA [NM_017675]	2.28	1.71E-30	26944	11742				
	PECAM1	platelet/endothelial cell adhesion molecule (CD31 anti	0.33	0	2059	6308				
	PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndr	2.07	4.80E-18	1346	648				
S√ IHC,M,PCR	POSTN	periostin, osteoblast specific factor (POSTN), mRNA [	53.68	0	36596	691	60.23	0	85449	1436
	PROS1	protein S (alpha) (PROS1), mRNA [NM_000313]	0.37	3.66E-09	298	785				
	PROS1	protein S (alpha) (PROS1), mRNA [NM_000313]	0.32	0	1388	4315				
	RDS	retinal degeneration, slow (RDS), mRNA [NM_000322	3.92	8.08E-09	666	165				
	ROBO1	roundabout, axon guidance receptor, homolog 1 (Dros	3.11	7.35E-31	1534	495	3.22	4.31E-24	1656	518
	ROM1	retinal outer segment membrane protein 1 (ROM1), m	0.37	5.75E-15	955	2618	0.40	2.83E-40	1015	2526
	SAA1	serum amyloid A1 (SAA1), transcript variant 1, mRNA	0.03	0	2033	78668	0.03	0	1919	72560
	SAA2	serum amyloid A2 (SAA2), mRNA [NM_030754]	0.05	0	2594	49704	0.05	0	2220	41872
	SAA4	serum amyloid A4, constitutive (SAA4), mRNA [NM_0	0.02	0	213	8743	0.02	0	211	9224

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	SLIT3	slit homolog 3 (Drosophila) (SLIT3), mRNA [NM_0030	3.95	0	14124	3576	2.49	1.51E-26	8137	3237
	STIM2	stromal interaction molecule 2 (STIM2), mRNA [NM_0	0.40	1.57E-06	1223	3239	0.46	2.51E-06	1256	2808
	TAOK2	TAO kinase 2 (TAOK2), mRNA [NM_016151]	2.07	0	58320	28140				
	THBS2	thrombospondin 2 (THBS2), mRNA [NM_003247]	6.57	1.21E-33	18698	2782	5.36	0	12664	2355
	XLKD1	extracellular link domain containing 1 (XLKD1), mRNA	0.07	0	654	9823	0.21	0	1845	8662

7) Proliferation

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ANAPC1	anaphase promoting complex subunit 1 (ANAPC1), m	0.47	2.30E-18	684	1454				
	ALS2CR19	amyotrophic lateral sclerosis 2 (juvenile) chromosome	0.46	0.00019	337	704	0.50	2.96E-08	304	599
	ANGPTL7	angiopoietin-like 7 (ANGPTL7), mRNA [NM_021146]	0.005	0	1284	274406	0.05	0	11741	233940
	BCAT1	branched chain aminotransferase 1, cytosolic (BCAT1	0.39	1.90E-18	2824	7216	0.35	1.76E-29	2542	7317
	BCL3	B-cell CLL/lymphoma 3 (BCL3), mRNA [NM_005178]	0.48	7.85E-17	5337	11201				
	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6	0.05	0	1735	35683	0.10	0	3027	30979
	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein) (BCL1	3.03	0	8619	2849				
	BCR	breakpoint cluster region (BCR), transcript variant 2, r	4.99	0	3447	688	3.58	1.52E-21	1876	508
	BCR		3.60	0	10584	2943				
	BIN1	bridging integrator 1 (BIN1), transcript variant 4, mRN	2.00	2.82E-10	1450	728				
S=R PCR	BMP7	bone morphogenetic protein 7 (osteogenic protein 1) (	16.33	0	2205	136	5.36	9.92E-13	552	99
	BMP8A	bone morphogenetic protein 8a (BMP8A), mRNA [NM	2.90	8.89E-17	47854	16023	2.31	3.31E-12	36604	15440
	CCL23	chemokine (C-C motif) ligand 23 (CCL23), transcript v	0.28	3.52E-09	225	794	0.43	1.35E-09	354	839
	CCNB2	cyclin B2 (CCNB2), mRNA [NM_004701]	3.27	0	2719	832	2.56	3.48E-42	1908	746
A√ IHC,M	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1) (CCN	3.06	1.22E-37	2463	802	2.41	2.61E-23	2102	867
A√ M	CCND2	cyclin D2 (CCND2), mRNA [NM_001759]	2.10	6.80E-12	1014	479	2.72	1.20E-11	913	346
	CCND2		5.16	5.81E-31	1799	350				
	CCNDBP1	cyclin D-type binding-protein 1 (CCNDBP1), transcript	0.30	4.55E-25	1804	5998	0.44	1.71E-10	2675	6135
	CCNG2	cyclin G2 (CCNG2), mRNA [NM_004354]	0.49	2.75E-16	3246	6722	0.41	2.62E-26	2393	5814
	CCNI	cyclin I (CCNI), mRNA [NM_006835]	0.45	5.96E-41	13757	30362				
	CCNL1	cyclin L1 (CCNL1), mRNA [NM_020307]	0.33	0	967	2924	0.33	5.61E-45	818	2498
	CCNL1		0.31	1.74E-34	2178	7150	0.32	1.09E-43	1962	6094
	CD248	CD248 antigen, endosialin (CD248), mRNA [NM_0204	4.03	1.10E-31	24947	6051	3.30	2.05E-34	18407	5512
	CDC14B	CDC14 cell division cycle 14 homolog B (S. cerevisiae	0.49	2.32E-11	2935	6114				
	CDC91L1	CDC91 cell division cycle 91-like 1 (S. cerevisiae) (CD	2.08	5.70E-21	7106	3401				
	CDK2AP1	CDK2-associated protein 1 (CDK2AP1), mRNA [NM_(	3.49	0	9509	2726	3.44	0	10106	2930
	CDK6	cyclin-dependent kinase 6 (CDK6), mRNA [NM_00125	2.14	1.82E-09	626	293	6.99	0	3144	447
	CDK6		6.46	0	3321	515				
	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDK	0.26	2.80E-45	863	3382	0.31	1.77E-39	986	3235
	CENPB	centromere protein B, 80kDa (CENPB), mRNA [NM_0	2.27	2.36E-16	1123	497				
	CGREF1	cell growth regulator with EF hand domain 1 (CGREF	4.57	0	1635	359	3.48	8.90E-24	1287	375
	CKS1B	CDC28 protein kinase regulatory subunit 1B (CKS1B)	0.49	1.68E-11	4803	9878				
	CLK1	CDC-like kinase 1 (CLK1), mRNA [NM_004071]	0.29	0	1087	3737	0.30	0	1107	3715
	CLK1		0.32	8.92E-22	2024	6304	0.35	5.48E-21	2077	6045
	CMIP	c-Maf-inducing protein (CMIP), transcript variant C-mi	3.31	3.53E-27	2456	736	4.07	2.43E-41	50177	12410
	CMIP		5.61	0	105445	18435				
A√ M	CRLF1	cytokine receptor-like factor 1 (CRLF1), mRNA [NM_0	0.27	0	4269	15813	0.31	0	4492	14588
A√ M	CSRP2	cysteine and glycine-rich protein 2 (CSRP2), mRNA [N	5.68	0	23708	4138	6.02	0	26372	4366
S√ M	DCN	decorin (DCN), transcript variant A1, mRNA [NM_001	0.39	1.34E-11	60964	157385	0.40	1.90E-15	54925	139728
	DCTN1	dynactin 1 (p150, glued homolog, Drosophila) (DCTN	2.52	1.90E-40	51651	20421				
	DLC1	deleted in liver cancer 1 (DLC1), transcript variant 3, r	4.19	0	7643	1822	2.27	4.16E-39	3105	1368
	DOCK9	dedicator of cytokinesis 9 (DOCK9), mRNA [NM_0152	0.34	6.82E-36	448	1313	0.49	7.88E-11	728	1478
	ECRG4	esophageal cancer related gene 4 protein (ECRG4), r	0.09	0	95	1070	0.12	1.91E-29	140	1100
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	0.13	0	628	5007	0.26	0	1125	4354

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	EDNRA	endothelin receptor type A (EDNRA), mRNA [NM_001	2.31	8.13E-06	772	326				
	EGFL6	EGF-like-domain, multiple 6 (EGFL6), mRNA [NM_01	3.02	2.72E-30	1099	365				
	EGFR	epidermal growth factor receptor (erythroblastic leuke	0.41	0.00052	257	658				
A√ PCR	ELN	elastin (supravalvular aortic stenosis, Williams-Beurer	2.53	3.22E-37	4214	1662				
	ELN		2.00	3.12E-43	78634	39240				
	EPS8	epidermal growth factor receptor pathway substrate 8	0.26	1.37E-31	2022	7828	0.61	3.38E-06	4557	7430
	EREG	epiregulin (EREG), mRNA [NM_001432]	3.05	1.18E-15	629	205	3.45	7.21E-16	537	155
			5.93	2.27E-14	1257	215				
	ERG	v-ets erythroblastosis virus E26 oncogene like (avian)	0.48	7.79E-22	656	1369				
	ERK8	mitogen-activated protein kinase 15 (MAPK15), mRNA	3.34	4.17E-38	1494	446				
	ERK8		2.98	0	4360	1460				
	ERN1	endoplasmic reticulum to nucleus signalling 1 (ERN1)	2.02	1.70E-20	1991	987				
	F2R	coagulation factor II (thrombin) receptor (F2R), mRNA	3.01	5.57E-13	871	292	2.68	6.69E-23	963	361
	FABP3	fatty acid binding protein 3, muscle and heart (mamma	0.50	0.02468	248	528				
A,S√ M	FAP	fibroblast activation protein, alpha (FAP), mRNA [NM_	52.70	0	56300	1063	40.41	0	41456	1023
	FAT	FAT tumor suppressor homolog 1 (Drosophila) (FAT),	2.89	1.40E-45	4138	1430	3.28	0	4241	1294
	FGF1	fibroblast growth factor 1 (acidic) (FGF1), transcript va	4.24	0	1627	384	3.49	1.67E-27	1720	495
	FGF7	fibroblast growth factor 7 (keratinocyte growth factor) (	0.46	1.54E-27	766	1683	0.32	1.40E-45	510	1616
	FGF7		0.29	2.64E-22	3824	13118	0.19	0	2448	12905
	FGFR1OP	FGFR1 oncogene partner (FGFR1OP), transcript varia	2.34	9.56E-13	697	297				
	FOSL1	FOS-like antigen 1 (FOSL1), mRNA [NM_005438]	2.13	7.5E-10	1587	736				
	G0S2	putative lymphocyte G0/G1 switch gene (G0S2), mRN	0.04	0	987	27098	0.05	0	1454	27317
	GHR	growth hormone receptor (GHR), mRNA [NM_000163	0.16	0	525	3281	0.15	0	422	2862
	GHR						0.44	1.68E-13	748	1658
	GMNN	geminin, DNA replication inhibitor (GMNN), mRNA [NI	0.27	7.45E-24	763	2837	0.33	9.32E-15	869	2711
	HK2	hexokinase 2 (HK2), mRNA [NM_000189]	0.43	5.64E-17	798	1863	0.28	0	503	1804
	HMGB2	high-mobility group box 2 (HMGB2), mRNA [NM_0021	0.20	0	2208	10877	0.18	0	1823	10074
A√ M,W,N	IGFBP6	insulin-like growth factor binding protein 6 (IGFBP6), r	0.09	0	380	4180	0.25	6.62E-35	1172	4684
	IGF2AS	insulin-like growth factor 2 antisense (IGF2AS), mRNA	2.11	4.82E-27	58563	27704				
	IGFL2	insulin growth factor-like family member 2 (IGFL2), mR	9.28	0	1256	136				
	IFITM1	interferon induced transmembrane protein 1 (9-27) (IF	0.33	0	6387	19636				
	IFNB1	interferon, beta 1, fibroblast (IFNB1), mRNA [NM_002	3.93	1.69E-37	1779	454	3.32	6.07E-08	1778	531
	IL6R	interleukin 6 receptor (IL6R), transcript variant 1, mRN	0.27	8.10E-26	401	1497	0.25	4.75E-30	391	1540
	INHBB	inhibin, beta B (activin AB beta polypeptide) (INHBB),	0.39	6.61E-31	1704	4376	0.30	3.39E-27	1254	4220
	INSIG1	insulin induced gene 1 (INSIG1), transcript variant 2, r	0.40	1.62E-27	578	1448				
	IRF2	interferon regulatory factor 2 (IRF2), mRNA [NM_0021	0.46	1.11E-11	465	1009				
	IRS1	insulin receptor substrate 1 (IRS1), mRNA [NM_00554	3.33	0	2352	706	2.85	2.80E-45	1796	632
	IRS2	insulin receptor substrate 2 (IRS2), mRNA [NM_00374	0.29	0	656	2298	0.25	0	574	2252
	JAK2	Janus kinase 2 (a protein tyrosine kinase) (JAK2), mR	0.37	6.45E-11	374	1017				
	JUN	v-jun sarcoma virus 17 oncogene homolog (avian) (JU	0.46	0	19284	42331				
	KEAP1	kelch-like ECH-associated protein 1 (KEAP1), transcri	22.79	0	145654	6363	12.33	0	41872	3400
	KLF11	Kruppel-like factor 11 (KLF11), mRNA [NM_003597]	0.44	1.99E-36	711	1604				
	KLF15	Kruppel-like factor 15 (KLF15), mRNA [NM_014079]	0.06	0	420	7099	0.05	0	381	6998
	KLF4	Kruppel-like factor 4 (gut) (KLF4), mRNA [NM_00423	0.39	1.61E-36	604	1565	0.37	1.33E-28	537	1468
	KLF5	Kruppel-like factor 5 (intestinal) (KLF5), mRNA [NM_0	0.14	1.36E-27	246	1790	0.21	0	315	1533
	LMO1	LIM domain only 1 (rhombotin 1) (LMO1), mRNA [NM_	3.31	0	10384	3142				
	LRP12	low density lipoprotein-related protein 12 (LRP12), mR	3.07	1.17E-28	1692	549	3.41	3.36E-44	1774	517
	LRP12		2.19	6.24E-17	1885	862	2.55	1.80E-31	2427	947
	LTBP2	latent transforming growth factor beta binding protein	0.38	3.97E-12	350	911				
	LZTS1	leucine zipper, putative tumor suppressor 1 (LZTS1),	4.86	9.00E-27	1363	278	11.63	0	3849	331
S√ M	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene ho	5.15	1.70E-31	22863	4306	6.55	1.54E-44	29490	4420
	MARK4	MAP/microtubule affinity-regulating kinase 4 (MARK4)	4.14	2.24E-27	10234	2427	3.61	2.85E-28	10009	2684

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	MATK	megakaryocyte-associated tyrosine kinase (MATK), tr	0.20	0	317	1576	0.19	0	306	1616
A√ M	MDK	midkine (neurite growth-promoting factor 2) (MDK), tra	5.72	0	20422	3560	2.19	0	7458	3399
	MTA1	metastasis associated 1 (MTA1), mRNA [NM_004689	2.10	2.56E-24	13619	6494				
	MTSS1	metastasis suppressor 1 (MTSS1), mRNA [NM_01475	0.06	0	308	4795	0.24	0	1117	4639
	MXI1	MAX interactor 1 (MXI1), transcript variant 2, mRNA [N	0.38	0	1877	4889	0.48	1.76E-37	2295	4811
S>R IHC.	MYC	v-myc myelocytomatosis viral oncogene homolog (avi	0.23	0	901	3971	0.29	1.77E-21	1390	4801
	NAP1L1	nucleosome assembly protein 1-like 1 (NAP1L1), tran	0.38	1.49E-12	5696	15278				
	NCK1	NCK adaptor protein 1 (NCK1), mRNA [NM_006153]	0.39	4.68E-27	1455	3765	0.46	0	1693	3677
	NDNL2	necdin-like 2 (NDNL2), mRNA [NM_138704]	2.11	0	5155	2437				
	NF2	neurofibromin 2 (bilateral acoustic neuroma) (NF2), tra	2.05	9.72E-09	8013	3760	2.37	5.02E-11	10628	4433
	NFIA	nuclear factor I/A (NFIA), mRNA [NM_005595]	0.49	2.71E-09	2368	4909	0.39	1.75E-16	1596	4117
	OSMR	oncostatin M receptor, mRNA (cDNA clone IMAGE:51	2.30	8.04E-23	1588	689	2.01	0.0001	1085	506
	P8	p8 protein (candidate of metastasis 1) (P8), mRNA [N	0.29	0	6713	23553	0.32	1.26E-44	7565	23440
	PA2G4	proliferation-associated 2G4, 38kDa (PA2G4), mRNA	0.44	6.31E-20	546	1237	0.48	1.28E-12	609	1251
	PA2G4		0.40	2.73E-22	1240	3110	0.49	1.85E-25	1541	3183
	PA2G4		0.48	3.60E-40	2390	4972				
	PARD6G	par-6 partitioning defective 6 homolog gamma (C. ele	3.84	2.32E-36	1119	292	2.84	1.31E-11	789	270
	PARD6G		5.28	0	15323	2887	3.71	0	10648	2856
	PBEF1	pre-B-cell colony enhancing factor 1 (PBEF1), transcri	0.13	0	1721	13112	0.12	0	1061	8553
	PBEF1		0.29	0	793	2784	0.10	0	1227	11912
	PBEF1		0.16	0	1150	7013	0.29	0	792	2718
	PDGFC	platelet derived growth factor C (PDGFC), mRNA [NM	2.19	6.40E-20	3513	1597	2.44	0	3722	1522
	PDGFRL	platelet-derived growth factor receptor-like (PDGFRL),	2.07	0	10733	5180	2.49	0	12350	4951
	PHF17	PHD finger protein 17 (PHF17), transcript variant S, m	0.15	0	888	5923	0.17	0	1111	6726
	PHF17		0.28	9.40E-10	148	519	0.39	0.00005	146	388
	PHF17		0.28	0.00001	111	387	0.27	1.64E-08	154	540
	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	0.08	0	3708	46224	0.13	0	6986	52284
	PLCE1	phospholipase C, epsilon 1 (PLCE1), mRNA [NM_016	0.39	3.10E-25	409	1040				
	PMP22	peripheral myelin protein 22 (PMP22), transcript varia	0.38	3.66E-23	9496	24739	0.40	6.56E-13	11892	30112
	PMS2L3	postmeiotic segregation increased 2-like 3 (PMS2L3),	2.05	2.62E-35	5298	2578				
	POLM	polymerase (DNA directed), mu (POLM), mRNA [NM_	2.12	2.90E-32	95537	45003				
	POLR3D	polymerase (RNA) III (DNA directed) polypeptide D, 44	3.31	0	6658	2013				
S√ IHC,M,PCR	POSTN	periostin, osteoblast specific factor (POSTN), mRNA [	53.68	0	36596	691	60.23	0	85449	1436
	PPP1CB	protein phosphatase 1, catalytic subunit, beta isoform	2.16	2.92E-07	2033	935				
	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15	0.40	0	4405	11039	0.41	0	3443	8480
S√ M	PRG4	proteoglycan 4 (PRG4), mRNA [NM_005807]	0.01	0	482	44080	0.12	0	4826	40787
A√ M	PRSS11	protease, serine, 11 (IGF binding) (PRSS11), mRNA [	10.16	4.1E-25	336708	30601	9.49	4.58E-33	287922	29149
	PSMD2	proteasome (prosome, macropain) 26S subunit, non-A	2.19	6.49E-37	2965	1354				
	PTTG1	pituitary tumor-transforming 1 (PTTG1), mRNA [NM_0	6.60	3.18E-37	25146	3703	4.53	1.38E-19	14803	3078
S√ M	PTN	pleiotrophin (heparin binding growth factor 8, neurite g	2.81	0	2525	899	3.69	0	3590	973
	PTN		3.83	0	24907	6515	4.78	0	29309	6130
	PTTG1	pituitary tumor-transforming 1 (PTTG1), mRNA [NM_0	6.60	3.18E-37	25146	3703	4.53	1.38E-19	14803	3078
	PYY	peptide YY (PYY), mRNA [NM_004160]	2.46	4.82E-31	6012	2444				
	RAB4B	RAB4B, member RAS oncogene family (RAB4B), mR	2.04	0.00009	589	294	6.30	0	4529	719
	RAB4B		7.66	7.99E-44	7188	929				
	RAB7B	RAB7B, member RAS oncogene family (RAB7B), mR	3.39	5.75E-18	1015	296	3.19	1.12E-23	967	306
	RAB7L1	RAB7, member RAS oncogene family-like 1 (RAB7L1	5.34	0	20566	3837	2.81	2.53E-26	11410	3977
	RAB15	RAB15, member RAS onocogene family (RAB15), mR	9.30	0	3957	425				
	RAB23	RAB23, member RAS oncogene family (RAB23), trans	2.01	1.72E-09	957	472	2.23	1.06E-11	1088	497
S√ M	RAB31	RAB31, member RAS oncogene family (RAB31), mRN	2.31	0	3893	1686	5.23	0	8902	1695
			2.72	0	4903	1801	3.71	0	7026	1895
	RABL2A	RAB, member of RAS oncogene family-like 2A (RABL	0.43	0	39552	91802	0.44	9.82E-38	34872	79770
	RARRES1	retinoic acid receptor responder (tazarotene induced)	0.19	0	1375	7208	0.26	0	1677	6560

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
S>A IHC,PCR	RB1	retinoblastoma 1 (including osteosarcoma) (RB1), mR	2.46	5.89E-17	6151	2449				
	RBL2	retinoblastoma-like 2 (p130) (RBL2), mRNA [NM_005	0.45	1.21E-08	702	1544				
	RBM9	RNA binding motif protein 9 (RBM9), mRNA [NM_014	2.41	0	4295	1777	2.13	9.32E-31	3682	1726
	REC8L1	REC8-like 1 (yeast) (REC8L1), mRNA [NM_005132]	5.42	3.17E-13	1900	336	2.98	1.72E-18	765	256
	REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (	0.32	1.54E-44	1770	5590	0.48	1.32E-15	2489	5152
	RFC3	replication factor C (activator 1) 3, 38kDa (RFC3), tran	0.44	3.61E-09	312	707	0.46	1.93E-09	298	647
	RHOQ	ras homolog gene family, member Q (RHOQ), mRNA	0.48	0.00007	353	720				
	RHOQ		0.46	2.72E-10	694	1505				
	RHOQ		0.50	4.74E-27	817	1642				
	RHOU	ras homolog gene family, member U (RHOU), mRNA	0.30	4.59E-13	216	703	0.35	2.59E-09	244	672
	RHOV	ras homolog gene family, member V (RHOV), mRNA [	4.03	0	9178	2279	2.76	0	5242	1896
	S100A11	S100 calcium binding protein A11 (calgizzarin) (S100A	0.36	0	4436	12460	0.40	9.20E-20	5010	12502
	S100B	S100 calcium binding protein, beta (neural) (S100B), r	0.32	1.64E-30	638	2013	0.30	0	638	2104
	SASH1	SAM and SH3 domain containing 1 (SASH1), mRNA [	0.21	0	2757	12841	0.24	0	2536	10612
	SASH1		0.22	6.10E-25	9263	43988	0.21	2.93E-33	8295	39542
	SDC1	syndecan 1 (SDC1), transcript variant 1, mRNA [NM_(	9.81	0	6976	700	4.68	3.17E-24	2507	533
	SHC1	SHC (Src homology 2 domain containing) transformin	2.15	4.28E-24	3620	1681				
	SLC19A2	solute carrier family 19 (thiamine transporter), membe	0.26	0	1599	6110	0.15	0	832	5596
	SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	0.32	0	695	2149	0.41	1.24E-20	927	2249
	SMAD3		0.28	1.33E-29	1180	4180	0.47	7.25E-26	2007	4272
S√ M	SPARC	secreted protein, acidic, cysteine-rich (osteonectin) (S	12.85	1.1E-44	440326	31435	13.33	0	433979	31064
	SPHK2	sphingosine kinase 2 (SPHK2), mRNA [NM_020126]	3.40	3.82E-17	12997	3825	2.76	0	7948	2880
	SSTR1	somatostatin receptor 1 (SSTR1), mRNA [NM_00104	2.43	0	5910	2435				
	ST13	suppression of tumorigenicity 13 (colon carcinoma) (H	0.49	1.38E-10	1092	2239	0.49	4.44E-15	947	1968
	ST13		0.47	4.43E-41	2983	6358	0.49	5.65E-20	2455	4997
	TACSTD2	tumor-associated calcium signal transducer 2 (TACST	0.49	6.55E-11	442	899	0.48	2.15E-10	479	1017
	TAOK2	TAO kinase 2 (TAOK2), mRNA [NM_016151]	2.07	0	58320	28140				
	TAX1BP3	Tax1 (human T-cell leukemia virus type I) binding prot	3.23	4.80E-39	1990	617	2.03	9.97E-17	1150	563
	TBC1D8	TBC1 domain family, member 8 (with GRAM domain)	0.29	1.08E-27	1157	4024	0.37	4.37E-23	1578	4241
S√ IHC, ELISA,M	TGFB2	transforming growth factor, beta 2 (TGFB2), mRNA [NM_003238]					3.06	1.82E-11	703	238
A√ M	TGFB3	transforming growth factor, beta 3 (TGFB3), mRNA [N	9.31	0	6452	684	8.77	0	6492	736
S√ IHC.	TGFB3						5.77	0.0002	305	64
S√ 2D-GE	TGFBI	transforming growth factor, beta-induced, 68kDa (TGF	4.56	1.2E-33	162242	35015	7.27	0	289821	39235
A>R W	TGFBR2	transforming growth factor, beta receptor II (70/80kDa	0.44	4.88E-15	382	861				
S√ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_00216(	2.48	0	5955	2403	8.99	0	41901	4677
	TNFAIP2	tumor necrosis factor, alpha-induced protein 2 (TNFAI	0.47	5.55E-39	5521	11687				
A√ M	TNFRSF11I	tumor necrosis factor receptor superfamily, member 1	0.30	0	557	1869	0.17	0	600	3514
	TNFRSF11B		0.26	0	894	3480	0.22	8.31E-32	379	1742
	TNFRSF12A	tumor necrosis factor receptor superfamily, member 1	4.56	5.51E-37	2834	618	4.80	6.36E-25	2906	602
	TNFRSF19	tumor necrosis factor receptor superfamily, member 1	20.61	3.90E-28	3316	158	6.43	2.25E-16	866	127
	TNFRSF19		2.38	0.0823	178	77				
	TNFRSF25	tumor necrosis factor receptor superfamily, member 2	3.18	4.98E-31	3460	1091	3.93	0	4083	1035
A√ M	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4	5.44	0	1755	323	7.01	0	2308	329
	TNFRSF7	tumor necrosis factor receptor superfamily, member 7	3.39	0	21208	6249	2.76	2.36E-18	13492	4887
	UBE2C	ubiquitin-conjugating enzyme E2C (UBE2C), transcrip	11.78	0	27438	2235	8.14	0	17445	2109
	UHRF1	ubiquitin-like, containing PHD and RING finger domain	4.56	8.76E-13	620	137				
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	5.48	0	2197	401	2.21	1.07E-09	649	289
	VEGF	vascular endothelial growth factor, mRNA (cDNA clone MGC:70609 IMAGE:6006890), com					0.48	5.79E-16	1188	2446
A√ M	WISP1	WNT1 inducible signaling pathway protein 1 (WISP1),	3.03	0	9133	3017	2.57	0.00001	4174	1751
A√ IHC,N,W,PCR	WT1	Wilms tumor 1 (WT1), transcript variant B, mRNA [NM	2.05	7.86E-06	695	344				
	WTAP	Wilms tumor 1 associated protein (WTAP), transcript	0.49	8.90E-11	426	859	0.49	1.30E-11	2345	4811
	WTAP		0.40	2.06E-21	1831	4645				
	ZFP36L2	zinc finger protein 36, C3H type-like 2 (ZFP36L2), mR	0.30	1.96E-29	11253	36799	0.31	1.12E-43	13537	43156

8) Cytoskeleton

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	APC	adenomatosis polyposis coli (APC), mRNA [NM_0000	2.16	1.20E-07	802	377				
	ACTB	actin, beta (ACTB), mRNA [NM_001101]	2.11	2.8E-19	35553	16645	2.01	0	53091	26364
	ACTL6B	actin-like 6B (ACTL6B), mRNA [NM_016188]	2.64	0	16004	6052	2.17	0	8315	3849
	ACTN1	actinin, alpha 1 (ACTN1), mRNA [NM_001102]	2.20	1.46E-15	3378	1532	2.14	2.18E-26	3488	1626
	ACTN3	actinin, alpha 3 (ACTN3), mRNA [NM_001104]	2.29	3.01E-06	701	312	2.05	0.00036	531	274
	CALD1	caldesmon 1 (CALD1), transcript variant 1, mRNA [NM	2.52	2.14E-28	1385	550				
	CALD1		2.41	3.22E-34	6018	2506				
	CALD1		2.39	3.83E-12	65096	27298				
	CDC42EP5	CDC42 effector protein (Rho GTPase binding) 5 (CDC	2.05	1.72E-20	20833	10114				
	CGNL1	cingulin-like 1 (CGNL1), mRNA [NM_032866]	0.23	3.40E-38	970	4222	0.48	2.23E-19	1766	3688
	CKAP4	cytoskeleton-associated protein 4 (CKAP4), mRNA [N	11.05	0	30543	2709	6.60	0	16551	2505
	CMIP	c-Maf-inducing protein (CMIP), transcript variant C-mi	3.31	3.53E-27	2456	736	4.07	2.43E-41	50177	12410
	CMIP		5.61	0	105445	18435				
	DLC1	deleted in liver cancer 1 (DLC1), transcript variant 3, r	4.19	0	7643	1822	2.27	4.16E-39	3105	1368
	DYSF	dysferlin, limb girdle muscular dystrophy 2B (autosom	0.44	1.48E-12	1222	2811	0.42	0	884	2127
	FSD1	fibronectin type III and SPRY domain containing 1 (FS	10.16	0	12113	1185	5.82	0	4401	753
	GEFT	RAC/CDC42 exchange factor (GEFT), transcript varia	0.37	2.26E-10	773	2110	0.45	3.07E-08	829	1828
	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypepti	3.14	6.26E-22	1077	344	4.90	8.06E-32	1832	378
			2.94	0	10769	3662	5.04	0	19513	3872
	MGC8685	tubulin, beta polypeptide paralog (RP11-506K6.1), mR	5.97	0	2153	361	12.51	0	7336	587
	MYH10	myosin, heavy polypeptide 10, non-muscle (MYH10),	3.53	0.0056	263	72				
	MYLIP	myosin regulatory light chain interacting protein (MYLI	0.33	7.39E-18	474	1426				
	MYO1B	myosin IB (MYO1B), mRNA [NM_012223]	3.07	0	4383	1423	4.87	0	7170	1467
	MYO1G	myosin IG (MYO1G), mRNA [NM_033054]	2.90	0	2737	942				
	MYO3A	myosin IIIA (MYO3A), mRNA [NM_017433]	3.19	1.21E-12	602	190	2.34	5.74E-06	565	254
	MYO7A	myosin VIIA (Usher syndrome 1B (autosomal recessiv	0.28	0	1720	6137	0.34	0	1798	5223
	MYOC	myocilin, trabecular meshwork inducible glucocorticoi	0.02	0	786	49667	0.07	0	3387	51303
	SSPN	sarcospan (Kras oncogene-associated gene) (SSPN),	2.28	3.86E-08	3787	1622	2.28	2.43E-11	4128	1826
A√ M	TRO	trophinin (TRO), transcript variant 3, mRNA [NM_0161	3.12	0	3220	1031	2.71	1.39E-43	2270	835
	TUBA1	tubulin, alpha 1 (testis specific) (TUBA1), mRNA [NM_	2.08	3.90E-24	12561	6022	2.35	0	13249	5633
	TUBA6	tubulin alpha 6 (TUBA6), mRNA [NM_032704]	2.26	1.21E-16	2690	1187	2.20	9.72E-41	2487	1130
	TUBA6	tubulin alpha 6 (TUBA6), mRNA [NM_032704]	2.33	0	4288	1838	2.47	0	4499	1824
	TUBA6	tubulin alpha 6 (TUBA6), mRNA [NM_032704]	2.15	3.62E-14	8531	3952	2.37	3.97E-18	9490	3928
	TUBA6	tubulin alpha 6 (TUBA6), mRNA [NM_032704]	2.04	1.12E-11	54810	27011				
	TUBB	tubulin, beta polypeptide (TUBB), mRNA [NM_178014	2.14	1.33E-11	1928	902				
	TUBB	tubulin, beta polypeptide (TUBB), mRNA [NM_178014	2.05	2.36E-27	9860	4799				
	TUBB2	tubulin, beta 2 (TUBB2), mRNA [NM_001069]	2.57	1.19E-31	3370	1309	3.35	0	4566	1363
	TUBB2	tubulin, beta 2 (TUBB2), mRNA [NM_001069]	2.06	4.80E-35	14200	6874	2.97	0	21249	7131
	TUBB3	tubulin, beta 3 (TUBB3), mRNA [NM_006086]	6.89	0	8233	1187	8.87	0	12157	1363
	TUBB4	tubulin, beta 4 (TUBB4), mRNA [NM_006087]	2.54	0.00005	602	245	2.84	7.11E-09	631	232
	TUBB6	tubulin, beta 6 (TUBB6), mRNA [NM_032525]	2.22	8.17E-35	3865	1743	2.77	0	4544	1638
	TUBB6	tubulin, beta 6 (TUBB6), mRNA [NM_032525]	2.02	1.46E-07	30253	14783	2.06	1.91E-08	31022	14838
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	5.48	0	2197	401	2.21	1.07E-09	649	289

9) Complement and coagulation cascades

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	C1RL	complement component 1, r subcomponent-like (C1R	0.21	0	675	3281	0.32	0	1033	3244
	C6	complement component 6 (C6), mRNA [NM_000065]	0.31	5.69E-10	176	570	0.35	3.06E-10	180	522
	C7	complement component 7 (C7), mRNA [NM_000587]					0.25	6.47E-22	217	854
	CFH	complement factor H (CFH), transcript variant 1, mRN	0.30	7.71E-10	563	1957	0.46	3.53E-11	1027	2260
	CFHL3	complement factor H-related 3 (CFHL3), mRNA [NM_	0.18	0	1660	9307	0.30	0	2345	7813
	CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repres					0.21	0	7749	36239
	DF	D component of complement (adipsin) (DF), mRNA [N	0.03	0	1567	46254	0.18	0	8228	45963
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	0.13	0	628	5007	0.26	0	1125	4354
	FANCE	Fanconi anemia, complementation group E (FANCE),	0.47	5.88E-18	1186	2509	0.45	4.45E-18	1119	2525
	F2R	coagulation factor II (thrombin) receptor (F2R), mRNA	3.01	5.57E-13	871	292	2.68	6.69E-23	963	361
	F2RL2	coagulation factor II (thrombin) receptor-like 2 (F2RL2	15.96	0	3448	213	5.05	1.89E-20	1023	196
	NM_001014	complement factor H (CFH), transcript variant 2, mRN	0.17	0	2372	13784	0.28	0	4265	15181
	PLG	plasminogen (PLG), mRNA [NM_000301]	5.56	0	43311	7793	3.39	0	24010	7092
	PTGER3	prostaglandin E receptor 3 (subtype EP3) (PTGER3),	3.17	1.01E-20	1600	507				
	RGC32	response gene to complement 32 (RGC32), mRNA [NM_014059]					0.32	0	1323	4177
							0.40	0	4193	10390
	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A (alpha	0.08	0	526	6291	0.09	0	544	5783
			0.15	0	426	2793	0.21	0	477	2342
	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha	4.73	4.20E-16	3499	728	2.80	9.34E-08	1838	668
	SERPINA1(	serine (or cysteine) proteinase inhibitor, clade A (alpha	2.59	3.16E-31	1487	575				
	SERPING1	serine (or cysteine) proteinase inhibitor, clade G (C1 in	0.26	0	1289	4950	0.34	3.82E-39	1445	4214
S√ M,W	SERPINH1	serine (or cysteine) proteinase inhibitor, clade H (heat	9.87	0	4017	406	5.35	2.61E-38	2103	395

10) Others

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ADRA1D	adrenergic, alpha-1D-, receptor (ADRA1D), mRNA [NI	2.15	1.52E-13	163378	76489				
	ARMC6	armadillo repeat containing 6 (ARMC6), mRNA [NM_(	2.16	3.09E-23	143428	66521				
	ARMC7	armadillo repeat containing 7 (ARMC7), mRNA [NM_(	5.82	1.97E-35	11705	1990	3.00	1.35E-23	3413	1095
	C1QTNF2	C1q and tumor necrosis factor related protein 2 (C1QT	2.08	1.72E-08	1165	553	2.04	4.28E-11	1151	556
A√ M	C1QTNF3	C1q and tumor necrosis factor related protein 3 (C1QT	11.89	0	6987	590	11.35	0	8272	727
	C1QTNF3		13.49	0	7533	560	13.14	0	7899	601
	C1QTNF6	C1q and tumor necrosis factor related protein 6 (C1QT	10.52	0	3173	301	6.53	0	2006	306
	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta (CEB	0.15	0	18519	125697	0.06	0	9484	148572
	CDO1	cysteine dioxygenase, type I (CDO1), mRNA [NM_001	0.03	0	777	30290	0.07	0	1715	24644
	CRISP2	cysteine-rich secretory protein 2 (CRISP2), mRNA [NM	15.55	0	14461	936	7.65	1.14E-38	3562	445
	CUTL2	cut-like 2 (Drosophila) (CUTL2), mRNA [NM_015267]	3.73	0	8809	2354	2.65	6.20E-25	6437	2408
	EBF	early B-cell factor (EBF), mRNA [NM_024007]	0.22	1.13E-22	242	1101	0.44	3.40E-17	545	1217
A√ M	EGFL3	EGF-like-domain, multiple 3 (EGFL3), mRNA [NM_00	2.68	2.70E-32	4527	1680	2.26	2.07E-41	3866	1708
	EGFL8	EGF-like-domain, multiple 8 (EGFL8), mRNA [NM_03	2.87	4.18E-25	33463	11545	2.31	1.64E-16	28862	12244
	FBL	fibrillarin (FBL), mRNA [NM_001436]	0.43	0	8560	19792	0.44	0	8539	19643
	FCN2	ficolin (collagen/fibrinogen domain containing lectin) 2	5.26	5.00E-12	1144	222	3.48	3.79E-08	651	202
	G1P2	interferon, alpha-inducible protein (clone IFI-15K) (G1I	11.97	0	129497	10868	7.08	0	55435	7835
	GPR32	G protein-coupled receptor 32 (GPR32), mRNA [NM_(	8.36	0	61762	7388	3.68	0	22196	6022
	GPR44	G protein-coupled receptor 44 (GPR44), mRNA [NM_(	4.52	1.09E-12	1207	273	2.69	6.88E-07	669	267
	HOMER1	homer homolog 1 (Drosophila) (HOMER1), mRNA [NM	8.18	1.18E-40	1471	178	3.75	5.80E-10	555	142
	HOMER3	homer homolog 3 (Drosophila) (HOMER3), mRNA [NM	2.25	9.08E-07	20525	8873	2.71	1.87E-12	24002	8610
	HMGN4	high mobility group nucleosomal binding domain 4 (HI	7.68	0	75900	10029	4.67	0	32987	7089
	KSP37	Ksp37 protein (KSP37), mRNA [NM_031950]	0.06	0	677	10918	0.09	0	223	2471
	KSP37	Ksp37 protein (KSP37), mRNA [NM_031950]	0.09	0	244	2828	0.07	0	747	11234
	LRRN1	leucine rich repeat neuronal 1 (LRRN1), mRNA [NM_(	26.52	3.92E-44	8611	313	12.28	0	3647	297
	MT1E	metallothionein 1E (functional) (MT1E), mRNA [NM_1	0.08	0	3279	41968	0.08	0	3447	41415
	MT1F	metallothionein 1F (functional) (MT1F), mRNA [NM_0(	0.10	0	1578	16487	0.09	0	1451	16283

			aggressive / reference				superficial / reference			
			ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	MT1G	metallothionein 1G (MT1G), mRNA [NM_005950]	0.49	2.4E-09	5030	10352	0.12	0	5451	45621
	MT1G		0.12	0	6055	49536	0.44	1.21E-20	4976	11330
	MT1H	metallothionein 1H (MT1H), mRNA [NM_005951]	0.22	2.1E-37	9213	42366	0.09	0	11311	127953
	MT1H		0.08	0	9452	118841	0.23	7.25E-21	10485	45262
	MT1K	metallothionein 1K (MT1K), mRNA [NM_176870]	0.06	0	6888	120968	0.07	0	7659	118336
	MT1L	H.sapiens mRNA for metallothionein isoform 1R. [X972	0.10	0	6213	64974	0.10	0	5744	58986
	MT1X	metallothionein 1X (MT1X), mRNA [NM_005952]	0.05	0	2270	43244	0.08	0	6336	83461
	MT1X		0.07	0	6067	83774	0.06	0	2870	47936
	MT2A	metallothionein 2A (MT2A), mRNA [NM_005953]	0.07	0	18135	261463	0.07	0	21591	312045
	MT2A		0.09	0	27444	329337	0.06	0	19012	317402
	MT2A		0.08	0	24253	307394	0.06	0	14725	257171
	MT1A	metallothionein 1A (functional) (MT1A), mRNA [NM_0	0.27	2.67E-27	19328	71913	0.26	4.45E-27	19352	73783
	MT1B	metallothionein 1B (functional) (MT1B), mRNA [NM_0	0.19	0	8004	42231	0.20	0	7785	38743
	MT1J	metallothionein 1J (MT1J), mRNA [NM_175622]	0.25	3.06E-34	1208	4921	0.22	0	1311	5876
	PGLYRP2	peptidoglycan recognition protein 2 (PGLYRP2), mRN	31.70	0	15454	489	22.90	0	9589	417
	PPIC	peptidylprolyl isomerase C (cyclophilin C) (PPIC), mRI	14.58	5.80E-40	51453	3318	8.43	3.46E-34	36495	4222
	PPIC		6.56	6.17E-40	16507	2479	4.08	9.35E-33	9176	2240
	POU4F2	POU domain, class 4, transcription factor 2 (POU4F2)	2.45	0	4067	1657				
	RAB11FIP1	RAB11 family interacting protein 1 (class I) (RAB11FIF	9.03	0	4919	545	3.62	1.63E-11	1316	341
	REC8L1	REC8-like 1 (yeast) (REC8L1), mRNA [NM_005132]	5.42	3.17E-13	1900	336	2.98	1.72E-18	765	256
	S100A8	S100 calcium binding protein A8 (calgranulin A) (S100	0.05	0	416	8235	0.04	0	267	7661
	S100A9	S100 calcium binding protein A9 (calgranulin B) (S100	0.06	0	126	1957	0.08	0	117	1559
	SMARCC2	SWI/SNF related, matrix associated, actin dependent	2.11	2.81E-40	234430	110879				
	SOD2	superoxide dismutase 2, mitochondrial (SOD2), mRNA	0.07	0	464	6867	0.15	0	451	3016
	SOD2		0.22	1.41E-25	644	2891	0.08	0	607	7158
	SOX3	SRY (sex determining region Y)-box 3 (SOX3), mRNA	6.64	0	87131	12896	4.90	2.42E-37	58713	11748
	TADA3L	transcriptional adaptor 3 (NGG1 homolog, yeast)-like	6.12	2.35E-39	7142	1152	3.31	3.12E-30	2703	806
	TXNIP	thioredoxin interacting protein (TXNIP), mRNA [NM_0	0.07	0	10673	160139	0.08	0	13384	161028
	UBOX5	U-box domain containing 5 (UBOX5), transcript varian	0.31	1.03E-16	241909	791541	0.34	6.18E-15	281568	826316
	URB	steroid sensitive gene 1 (URB), transcript variant 1, m	13.49	0	6181	461	12.12	0	6165	511
	URB		11.32	0	1502	134	5.79	2.26E-16	770	140
	VIT	vitrin (VIT), mRNA [NM_053276]	0.11	0	263	2319	0.19	0	405	2134

Gene list B: Genes differentially expressed between aggressive and superficial fibromatosis

For each gene, its expression ratios aggr/ref and super/ref are also shown, if they fulfill the stringent selection criteria for significant differential gene expression. Therefore, no numbers in the columns Aggr/Ref and/or Super/Ref indicate no statistically signifcant difference in the expressions of the corresponding gene.

The different colors in the front column indicate the differential expression of the corresponding gene between the three tissues:

Aggr > Ref > Super
Aggr > Super > Ref
Aggr < Super < Ref

Aggr > Super, Ref
Aggr < Super, Ref

Aggr ≠ Super

Super > Ref > Aggr
Super > Aggr > Ref
Super < Aggr < Ref

Super > Aggr, Ref
Super < Aggr, Ref

Genes whose differential expressions between fibromatoses and reference tissue (as it is mostly the case) or between the the two types of fibromatoses (seldom) have been described in the literature are marked with the according informations in the first column. Abbreviations:

A	**Aggressive** fibromatosis
S	**Superficial** fibromatosis
R	**Reference** fibrous tissue
√	Differential expression described in the **literature** could be **confirmed** by own results
no √	Differential expression described in the **literature could not be confirmed**, the result published in the literature is indicated, e.g. A = R
IHC.	Result published in the literature obtained by **immunohistochemical** analysis
M	Result published in the literature obtained by **microarray** gene expression analysis
W	Result published in the literature obtained by **Western blot** analysis
N	Result published in the literature obtained by **Northern blot** analysis
ELISA	Result published in the literature obtained by **ELISA** analysis
2D-GE	Result published in the literature obtained by **two-dimensional gel electrophoresis**, followed by **mass-spectrometry** (MS) analysis
PCR	Result published in the literature obtained by **RT-PCR** or **real-time RT-PCR**

1) Wnt signalling pathway

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	APC2	adenomatosis polyposis coli 2 (APC2), mRNA [NM_005883]	0.26	8.35E-13	174	709	7.02	8.65E-19	741	104				
							3.29	4.20E-45	11283	3425				
	AXIN2	axin 2 (conductin, axil) (AXIN2), mRNA [NM_004655]	0.48	2.87E-07	330	702	2.32	0.00002	711	312	2.18	0.00006	1179	564
			0.36	4.59E-21	1247	3478	5.54	5.43E-33	3650	661				
	CD44	CD44 antigen (homing function and Indian blood group system) (CD44), transcript variant 1, mRNA [NM	2.52	2.66E-37	14277	5645	0.29	1.37E-29	5892	20369				
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.36	1.57E-24	2411	6778					0.34	7.99E-22	3162	9385
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.33	2.85E-24	4793	14540					0.23	6.16E-25	5002	21658
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.37	1.66E-10	12641	34956					0.25	1.29E-13	13257	53481
	DKK2	dickkopf homolog 2 (Xenopus laevis) (DKK2), mRNA [NM_014421]	0.37	1.15E-18	279	755	2.40	3.08E-09	748	315				
			0.39	7.87E-35	568	1451	2.80	1.19E-27	1557	558				
	DKK3	dickkopf homolog 3 (Xenopus laevis) (DKK3), mRNA [NM_015881]	5.12	0	44149	8622					2.57	5.36E-32	9506	3729
			5.36	0	36184	6749					3.24	0	29583	9072
			3.40	4.76E-44	9139	2689					2.96	0	40095	13548
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	2.10	2.24E-22	1193	568	0.13	0	628	5007	0.26	0	1125	4354
	FGF18	fibroblast growth factor 18 (FGF18), transcript variant 1, mRNA [NM_003862]	3.05	0.00002	986	348					3.55	4.64E-30	1118	318
	FRZB	frizzled-related protein (FRZB), mRNA [NM_001463]	0.28	6.13E-33	532	1890					0.21	3.34E-35	526	2460
											0.30	1.85E-23	733	2484
	FST	follistatin (FST), transcript variant FST344, mRNA [NM_013409]	0.15	0	470	3057	3.95	0	3536	893				
	FZD2	frizzled homolog 2 (Drosophila) (FZD2), mRNA [NM_001466]	0.49	1.72E-09	473	980	4.42	2.77E-24	1038	237				
	FZD8	frizzled homolog 8 (Drosophila) (FZD8), mRNA [NM_031866]	3.40	4.22E-25	946	281	0.40	5.93E-09	276	706				
	GREM1	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis) (GREM1), mRNA [NM_013372]	0.14	9.75E-30	823	5990	5.54	0	6083	1090				
A=R PCR	LEF1	lymphoid enhancer-binding factor 1 (LEF1), mRNA [NM_016269]	0.39	1.19E-34	1327	3422	7.67	0	3569	467	3.11	1.69E-13	1260	392
	MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA [NM_000245]	3.49	0	4439	1270	0.32	0	1270	3907				
A>R M.PCR	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase) (MMP3), mRNA [NM_002422]	0.08	2.40E-29	54	651	0.01	0	3050	286788	0.01	0	3432	279991
	PITX2	paired-like homeodomain transcription factor 2 (PITX2), transcript variant 2, mRNA [NM_153426]	0.12	0	373	3142	8.89	0	3500	394				
AvR √ IHC,N,W	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), m	0.25	7.43E-15	150	606	2.83	1.27E-18	713	252				
	SFRP2	secreted frizzled-related protein 2 (SFRP2), mRNA [NM_003013]	0.35	0	2281	6510	10.58	0	26378	2490	3.66	0	1964	537
			0.54	5.51E-28	14362	26469	10.13	0	7482	740	2.91	0	3466	1192
			0.46	5.28E-24	3671	8022	5.73	0	7945	1388	5.98	0	11575	1934
	SFRP4	secreted frizzled-related protein 4 (SFRP4), mRNA [NM_003014]	2.69	0	71925	26775	16.57	0	29554	1781	30.97	0	73816	2390
	SP5	Sp5 transcription factor (SP5), mRNA [NM_001003845]	0.18	7.79E-40	313	1811	4.65	0	1989	429				
		Sp5 transcription factor (SP5), mRNA [NM_001003845]	0.11	2.58E-31	238	2194	5.18	5.17E-16	2214	428				
AvR √ PCR	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box) (TCF7L2), mRNA [NM_030756]	0.38	1.94E-08	221	563					0.36	1.45E-09	229	651
	TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) (TLE1), mRNA [NM_005077]	0.43	8.83E-25	742	1737								
	TNFRSF19	tumor necrosis factor receptor superfamily, member 19 (TNFRSF19), transcript variant 2, mRNA [NM_1	0.32	7.05E-33	904	2779	20.61	3.90E-28	3316	158	6.43	2.25E-16	866	127
		tumor necrosis factor receptor superfamily, member 19 (TNFRSF19), transcript variant 1, mRNA [NM_018647]					2.38	0.0823	178	77				

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	WIF1	WNT inhibitory factor 1 (WIF1), mRNA [NM_007191]	0.05	0	160	3201	14.17	0	2929	206				
	WISP2	WNT1 inducible signaling pathway protein 2 (WISP2), mRNA [NM_003881]	4.11	1.40E-32	29137	6995	0.23	9.48E-29	5443	23926				
	WNT2	wingless-type MMTV integration site family member 2 (WNT2), mRNA [NM_003391]	0.47	4.88E-09	441	959	3.43	7.80E-13	975	289				
	WNT4	wingless-type MMTV integration site family, member 4 (WNT4), mRNA [NM_030761]	0.50	4.48E-16	1843	3704	2.85	1.33E-39	3693	1293				
AvR √ M	WNT5A	wingless-type MMTV integration site family, member 5A (WNT5A), mRNA [NM_003392]	0.14	0	849	5937	14.40	0	5461	378	2.27	4.47E-09	657	295

2) TGFβ signalling pathway

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
AvR √ M	ASPN	asporin (LRR class 1) (ASPN), mRNA [NM_017680]	2.92	5.51E-14	104503	34787	14.89	0	36546	2424	27.27	0	91077	3308
	BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) (BAMBI), mRNA [NM_012342]	3.11	0	5244	1685					3.73	3.34E-43	4795	1283
S=R PCR	BMP7	bone morphogenetic protein 7 (osteogenic protein 1) (BMP7), mRNA [NM_001719]	0.31	6.31E-44	638	2062	16.33	0	2205	136	5.36	9.92E-13	552	99
	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C), mRNA [NM_000076]	2.27	3.55E-19	14786	6496								
	CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4) (CDKN2D), transcript variant 1, mRNA [NM_0	1.92	0	5936	3092								
	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase (CILP), mRNA [NM_003613]	4.23	3.99E-30	13165	3067	0.04	0	3778	87300	0.17	2.75E-22	12084	74050
A>R M	COMP	cartilage oligomeric matrix protein (COMP), mRNA [NM_000095]	3.53	0	50880	14435	0.12	7.40E-43	15337	131654				
			5.15	4.95E-40	75737	14436	0.18	0	17114	96665				
	EGR1	early growth response 1 (EGR1), mRNA [NM_001964]	0.39	1.00E-33	12036	30703					0.46	3.95E-15	10223	22395
	FST	follistatin (FST), transcript variant FST344, mRNA [NM_013409]	0.15	0	470	3057	3.95	0	3536	893				
	FSTL3	follistatin-like 3 (secreted glycoprotein) (FSTL3), mRNA [NM_005860]	0.41	7.45E-08	208	509	2.04	0.00002	531	263				
	GDF10	growth differentiation factor 10 (GDF10), mRNA [NM_004962]	2.18	5.68E-17	1634	742								
	GREM1	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis) (GREM1), mRNA [NM_013372]	0.14	9.75E-30	823	5990	5.54	0	6083	1090				
	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein (ID4), mRNA [NM_001546]	0.25	0	681	2777					0.33	0	636	1910
	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide) (INHBA), mRNA [NM_002192]	0.2	0	4'082	20'587	4.57	0	22595	4945				
	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3) (LGALS3), mRNA [NM_002306]	2.06	0	22673	10983	0.31	0	11067	35700				
	OGN	osteoglycin (osteoinductive factor, mimecan) (OGN), transcript variant 1, mRNA [NM_033014]	0.46	3.55E-23	3863	8405	16.59	0	8686	524	7.88	0	3775	480
	PITX2	paired-like homeodomain transcription factor 2 (PITX2), transcript variant 2, mRNA [NM_153426]	0.12	0	373	3142	8.89	0	3500	394				
	THBS3	thrombospondin 3 (THBS3), mRNA [NM_007112]	2.17	1.59E-18	54594	24955								
	THBS4	thrombospondin 4 (THBS4), mRNA [NM_003248]	7.99	2.8E-45	67565	8263	0.31	1.44E-15	6783	21857	2.59	4.00E-07	65044	23708

3) PI3K-AKT signalling pathway

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	AREG	amphiregulin (schwannoma-derived growth factor) (AREG), mRNA [NM_001657]	0.07	0	598	8751	9.51	0	8522	881				
	FGF18	fibroblast growth factor 18 (FGF18), transcript variant 1, mRNA [NM_003862]	3.05	0.00002	986	348					3.55	4.64E-30	1118	318
AvR √ M,W,N	IGFBP6	insulin-like growth factor binding protein 6 (IGFBP6), mRNA [NM_002178]	3.33	1.69E-25	1118	339	0.09	0	380	4180	0.25	6.62E-35	1172	4684
	IGFBP7	insulin-like growth factor binding protein 7 (IGFBP7), mRNA [NM_001553]	2.42	0	174079	71860					2.65	2.79E-19	153744	57411
	IGFL2	insulin growth factor-like family member 2 (IGFL2), mRNA [NM_001002915]	0.19	6.57E-16	225	1117	9.28	0	1256	136				
	IL13RA1	interleukin 13 receptor, alpha 1 (IL13RA1), mRNA [NM_001560]	2.11	4.60E-10	26206	12259	0.36	2.33E-09	12656	36105				
	IL17RB	interleukin 17 receptor B (IL17RB), transcript variant 2, mRNA [NM_172234]	0.45	5.44E-14	419	949								
	MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA [NM_000245]	3.49	0	4439	1270	0.32	0	1270	3907				
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide (PDGFRB), mRNA [NM_002609]	2.06	2.73E-19	38724	18703					2.32	5.85E-18	33903	14521

4) Extracellular matrix (ECM)

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ADAMTS1	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1),	0.16	0	779	4846	2.24	1.74E-25	4604	2051	0.45	1.98E-18	846	1849
			0.22	0	1021	4714	2.39	2.92E-25	5726	2390	0.33	0	719	2196
	ADAMTS4	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 4 (ADAMTS4),	0.48	3.87E-42	5291	11115								
	ADAMTS9	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9 (ADAMTS9),	0.33	1.91E-15	373	1114	3.39	6.16E-34	1189	351				
			0.45	1.02E-25	669	1505	2.15	5.39E-08	1456	684				
			0.17	0	2494	14527	5.07	0	15164	2987				
	AGC1	aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan, antigen identified by mo	3.77	0	49888	13236					5.74	0	43167	7532
	ANGPTL7	angiopoietin-like 7 (ANGPTL7), mRNA [NM_021146]	66.93	0	12779	192	0.005	0	1284	274406	0.05	0	11741	233940
AvR √ M	ASPN	asporin (LRR class 1) (ASPN), mRNA [NM_017680]	2.92	5.51E-14	104503	34787	14.89	0	36546	2424	27.27	0	91077	3308
	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase (CILP), mRNA [NM_003613]	4.23	3.99E-30	13165	3067	0.04	0	3778	87300	0.17	2.75E-22	12084	74050
AvR √ M	CLEC3B	C-type lectin domain family 3, member B (CLEC3B), mRNA [NM_003278]	3.23	0	18295	5652	0.38	1.08E-30	5534	14635				
S<R M														
AvR √ M	COL11A1	collagen, type XI, alpha 1 (COL11A1), transcript variant B, mRNA [NM_080629]	0.29	0	3126	10831	16.45	0	11610	708	5.43	0	3027	556
	COL17A1	collagen, type XVII, alpha 1 (COL17A1), transcript variant long, mRNA [NM_000494]	3.51	3.46E-13	507	145								
SvR √ IHC.	COL4A5	collagen, type IV, alpha 5 (Alport syndrome) (COL4A5), transcript variant 2, mRNA [NM_033380]	0.28	1.47E-24	224	797	2.30	3.38E-13	805	351	4.00	0	2598	649
							2.32	9.87E-21	1315	564				
							7.93	0	7045	883				
AvR √ M	COL5A1	collagen, type V, alpha 1 (COL5A1), mRNA [NM_000093]	0.38	1.30E-20	2185	5709	12.82	0	53506	4187	7.85	0	23475	3005
							28.83	0	5889	206	15.60	0	2091	134
	COL8A2	collagen, type VIII, alpha 2 (COL8A2), mRNA [NM_005202]	2.93	5.32E-33	15516	5262	2.20	5.66E-15	4848	2176	5.85	0	14811	2511
A>R M	COMP	cartilage oligomeric matrix protein (COMP), mRNA [NM_000095]	3.53	0	50880	14435	0.12	7.40E-43	15337	131654				
			5.15	4.95E-40	75737	14436	0.18	0	17114	96665				

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.36	1.57E-24	2411	6778					0.34	7.99E-22	3162	9385
			0.33	2.85E-24	4793	14540					0.23	6.16E-25	5002	21658
			0.37	1.66E-10	12641	34956					0.25	1.29E-13	13257	53481
AvR √ M	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), transcript variant 1, mRNA [NM_004	2.72	1.44E-28	10928	3990	0.33	5.54E-13	4514	13834				
	EGR1	early growth response 1 (EGR1), mRNA [NM_001964]	0.39	1.00E-33	12036	30703					0.46	3.95E-15	10223	22395
	FNDC1	Fibronectin type III domain containing 1	3.35	0	28913	8642								
	HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1, mRNA (cDNA clone MGC:71688 IMAGE:3034	0.25	4.57E-16	221	857	5.11	3.26E-06	637	115				
	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3) (LGALS3), mRNA [NM_002306]	2.06	0	22673	10983	0.31	0	11067	35700				
S>R 2D-GE	MFAP4	microfibrillar-associated protein 4 (MFAP4), mRNA [NM_002404]	0.31	1.28E-25	13129	41989	3.87	2.37E-21	44700	11483				
AvR √ PCR	MMP1	matrix metalloproteinase 1 (interstitial collagenase) (MMP1), mRNA [NM_002421]	0.39	8.22E-27	1098	2832	3.40	0	3132	923				
AvR √ PCR	MMP11	matrix metalloproteinase 11 (stromelysin 3) (MMP11), mRNA [NM_005940]	0.30	0	6151	20758	18.57	0	21077	1139	8.93	0	6272	693
	MMP19	matrix metalloproteinase 19 (MMP19), transcript variant rasi-6, mRNA [NM_022791]	0.32	1.68E-30	1252	3972	10.78	0	3898	362	3.43	4.58E-21	1259	360
	MMP23B	matrix metalloproteinase 23B (MMP23B), mRNA [NM_006983]	0.40	0	1706	4317	6.09	0	4386	719	2.75	1.54E-42	1210	439
A>R M,PCR	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase) (MMP3), mRNA [NM_002422]	0.08	2.40E-29	54	651	0.01	0	3050	286788	0.01	0	3432	279991
A<R M	NID2	nidogen 2 (osteonidogen) (NID2), mRNA [NM_007361]	2.02	1.26E-13	6022	2972	7.30	0	2909	396	12.24	0	5659	460
	NOV	nephroblastoma overexpressed gene (NOV), mRNA [NM_002514]	8.90	0	9069	1018					9.90	0	8761	886
	OGN	osteoglycin (osteoinductive factor, mimecan) (OGN), transcript variant 1, mRNA [NM_033014]	0.46	3.55E-23	3863	8405	16.59	0	8686	524	7.88	0	3775	480
	PLAT	plasminogen activator, tissue (PLAT), transcript variant 1, mRNA [NM_000930]	0.16	0	2024	12582	2.01	2.33E-08	13121	6523	0.33	6.95E-27	1738	5366
	PLAU	plasminogen activator, urokinase (PLAU), mRNA [NM_002658]	2.50	1.11E-30	18274	7260					4.10	0	17120	4155
SvR √ M	PRG4	proteoglycan 4 (PRG4), mRNA [NM_005807]	18.71	0	4629	246	0.01	0	482	44080	0.12	0	4826	40787
	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5 (SERPI	4.02	0.04679	659	147	4.73	4.20E-16	3499	728	2.80	9.34E-08	1838	668
A>R M	SPARCL1	SPARC-like 1 (mast9, hevin) (SPARCL1), mRNA [NM_004684]	2.46	1.35E-34	153223	62080								
AvR √ M	SPON2	spondin 2, extracellular matrix protein (SPON2), mRNA [NM_012445]	3.12	7.47E-20	282110	88387	9.59	1.19E-41	103308	10375	23.98	0	278300	11395
	THBS3	thrombospondin 3 (THBS3), mRNA [NM_007112]	2.17	1.59E-18	54594	24955								
	THBS4	thrombospondin 4 (THBS4), mRNA [NM_003248]	7.99	2.8E-45	67565	8263	0.31	1.44E-15	6783	21857	2.59	4.00E-07	65044	23708
SvR √ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_002160]	5.24	0	40057	7601	2.48	0	5955	2403	8.99	0	41901	4677
	TNXB	tenascin XB (TNXB), transcript variant XB, mRNA [NM_019105]	5.99	0	22076	3653	0.13	0	3271	25201				

5) ECM-receptor interaction

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ACTN2	actinin, alpha 2 (ACTN2), mRNA [NM_001103]	0.06	0	64	1129	16.32	1.67E-42	1217	74				
	CD34	CD34 antigen (CD34), mRNA [NM_001773]	2.46	1.56E-23	8200	3313	0.38	0	3624	9566				
	CD44	CD44 antigen (homing function and Indian blood group system) (CD44), transcript variant 1, mRNA [NM	2.52	2.66E-37	14277	5645	0.29	1.37E-29	5892	20369				
AvR √ M	COL11A1	collagen, type XI, alpha 1 (COL11A1), transcript variant B, mRNA [NM_080629]	0.29	0	3126	10831	16.45	0	11610	708	5.43	0	3027	556
	COL17A1	collagen, type XVII, alpha 1 (COL17A1), transcript variant long, mRNA [NM_000494]	3.51	3.46E-13	507	145								
SvR √ IHC.	COL4A5	collagen, type IV, alpha 5 (Alport syndrome) (COL4A5), transcript variant 2, mRNA [NM_033380]	0.28	1.47E-24	224	797	2.30	3.38E-13	805	351	4.00	0	2598	649
							2.32	9.87E-21	1315	564				
							7.93	0	7045	883				
AvR √ M	COL5A1	collagen, type V, alpha 1 (COL5A1), mRNA [NM_000093]	0.38	1.30E-20	2185	5709	12.82	0	53506	4187	7.85	0	23475	3005
							28.83	0	5889	206	15.60	0	2091	134
	COL8A2	collagen, type VIII, alpha 2 (COL8A2), mRNA [NM_005202]	2.93	5.32E-33	15516	5262	2.20	5.66E-15	4848	2176	5.85	0	14811	2511
A>R M	COMP	cartilage oligomeric matrix protein (COMP), mRNA [NM_000095]	3.53	0	50880	14435	0.12	7.40E-43	15337	131654				
			5.15	4.95E-40	75737	14436	0.18	0	17114	96665				
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.36	1.57E-24	2411	6778					0.34	7.99E-22	3162	9385
			0.33	2.85E-24	4793	14540					0.23	6.16E-25	5002	21658
			0.37	1.66E-10	12641	34956					0.25	1.29E-13	13257	53481
	EDIL3	EGF-like repeats and discoidin I-like domains 3 (EDIL3), mRNA [NM_005711]	2.78	1.03E-41	21930	7892	8.77	0	8573	968	20.14	0	18664	922
	FIGF	c-fos induced growth factor (vascular endothelial growth factor D) (FIGF), mRNA [NM_004469]	0.48	1.84E-22	824	1716								
	FLRT3	fibronectin leucine rich transmembrane protein 3 (FLRT3), transcript variant 2, mRNA [NM_198391]	0.49	1.1E-12	397	824	2.24	9.58E-08	763	345				
	FNDC1	Fibronectin type III domain containing 1	3.35	0	28913	8642								
	ITGA8	integrin, alpha 8 (ITGA8), mRNA [NM_003638]	3.84	7.06E-41	2259	590					6.47	0	2527	391
	ITGA9	integrin, alpha 9 (ITGA9), mRNA [NM_002207]	2.42	3.60E-12	1010	406					3.57	6.80E-13	921	247
	MAPK10	mitogen-activated protein kinase 10 (MAPK10), transcript variant 3, mRNA [NM_138980]	0.44	0.00005	238	503	2.09	7.54E-06	510	246				
	MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA [NM_000245]	3.49	0	4439	1270	0.32	0	1270	3907				
S>R 2D-GE	MFAP4	microfibrillar-associated protein 4 (MFAP4), mRNA [NM_002404]	0.31	1.28E-25	13129	41989	3.87	2.37E-21	44700	11483				
	MYL1	myosin, light polypeptide 1, alkali; skeletal, fast (MYL1), transcript variant 3f, mRNA [NM_079422]	0.12	1.24E-29	118	1031	4.51	3.12E-19	1018	229				
	MYLK	myosin, light polypeptide kinase (MYLK), transcript variant 1, mRNA [NM_053025]	0.50	5.35E-43	2955	5636	2.35	1.52E-20	5877	2481				
							2.03	3.58E-11	24837	12243				
	NEDD9	neural precursor cell expressed, developmentally down-regulated 9 (NEDD9), mRNA [NM_006403]	2.24	1.42E-10	692	313	0.45	2.92E-07	350	761				
A<R M	NID2	nidogen 2 (osteonidogen) (NID2), mRNA [NM_007361]	2.02	1.26E-13	6022	2972	7.30	0	2909	396	12.24	0	5659	460
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide (PDGFRB), mRNA [NM_002609]	2.06	2.73E-19	38724	18703					2.32	5.85E-18	33903	14521
	PPP1R12B	protein phosphatase 1, regulatory (inhibitor) subunit 12B (PPP1R12B), transcript variant 2, mRNA [NM_	0.44	9.97E-12	5587	12707								
	SDC1	syndecan 1 (SDC1), transcript variant 1, mRNA [NM_001006946]	0.34	3.75E-34	2339	6979	9.81	0	6976	700	4.68	3.17E-24	2507	533
	SORBS1	sorbin and SH3 domain containing 1 (SORBS1), mRNA [NM_015385]	0.32	2.25E-38	659	2046					0.40	1.45E-12	1055	2618
											0.20	7.39E-23	639	3261
	SPARCL1	SPARC-like 1 (mast9, hevin) (SPARCL1), mRNA [NM_004684]	2.46	1.35E-34	153223	62080								

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
AvR √ M	SPON2	spondin 2, extracellular matrix protein (SPON2), mRNA [NM_012445]	3.12	7.47E-20	282110	88387	9.59	1.19E-41	103308	10375	23.98	0	278300	11395
	THBS3	thrombospondin 3 (THBS3), mRNA [NM_007112]	2.17	1.59E-18	54594	24955								
	THBS4	thrombospondin 4 (THBS4), mRNA [NM_003248]	7.99	2.8E-45	67565	8263	0.31	1.44E-15	6783	21857	2.59	4.00E-07	65044	23708
SvR √ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_002160]	5.24	0	40057	7601	2.48	0	5955	2403	8.99	0	41901	4677
A<R M	TNFAIP6	tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA [NM_007115]	2.52	3.04E-25	2109	830					2.78	8.97E-39	2084	749
	TNXB	tenascin XB (TNXB), transcript variant XB, mRNA [NM_019105]	5.99	0	22076	3653	0.13	0	3271	25201				
	TTN	titin (TTN), transcript variant novex-3, mRNA [NM_133379]	0.11	0	633	5824	11.65	0	5738	492				
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	0.39	1.28E-32	802	2046	5.48	0	2197	401	2.21	1.07E-09	649	289

6) Adherens junction and cell adhesion molecules (CAMs)

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1) (AOC3), mRNA [NM_003734]	0.48	5.68E-11	1117	2339								
	CD34	CD34 antigen (CD34), mRNA [NM_001773]	2.46	1.56E-23	8200	3313	0.38	0	3624	9566				
	CD44	CD44 antigen (homing function and Indian blood group system) (CD44), transcript variant 1, mRNA [NM	2.52	2.66E-37	14277	5645	0.29	1.37E-29	5892	20369				
	CD9	CD9 antigen (p24) (CD9), mRNA [NM_001769]	2.36	3.17E-42	4833	2044	0.35	0	2124	6096				
	CDH13	cadherin 13, H-cadherin (heart) (CDH13), mRNA [NM_001257]	4.69	1.40E-45	8831	1877	0.49	6.95E-17	1565	3222	2.24	0	9760	4354
	CPXM2	carboxypeptidase X (M14 family), member 2 (CPXM2), mRNA [NM_198148]	4.43	0	8399	1896					2.63	7.45E-23	8418	3173
	FLRT3	fibronectin leucine rich transmembrane protein 3 (FLRT3), transcript variant 2, mRNA [NM_198391]	0.49	1.1E-12	397	824	2.24	9.58E-08	763	345				
A=R PCR	LEF1	lymphoid enhancer-binding factor 1 (LEF1), mRNA [NM_016269]	0.39	1.19E-34	1327	3422	7.67	0	3569	467	3.11	1.69E-13	1260	392
	MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA [NM_000245]	3.49	0	4439	1270	0.32	0	1270	3907				
S>R 2D-GE	MFAP4	microfibrillar-associated protein 4 (MFAP4), mRNA [NM_002404]	0.31	1.28E-25	13129	41989	3.87	2.37E-21	44700	11483				
	PCDH7	BH-protocadherin (brain-heart) (PCDH7), transcript variant c, mRNA [NM_032457]	2.52	7.67E-08	831	313	2.66	4.57E-23	938	353	2.75	2.98E-30	1160	423
											19.74	8.38E-25	740	40
											2.29	0.11056	140	60
	SORBS1	sorbin and SH3 domain containing 1 (SORBS1), mRNA [NM_015385]	0.32	2.25E-38	659	2046					0.40	1.45E-12	1055	2618
											0.20	7.39E-23	639	3261
AvR √ PCR	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box) (TCF7L2), mRNA [NM_030756]	0.38	1.94E-08	221	563					0.36	1.45E-09	229	651
	THBS3	thrombospondin 3 (THBS3), mRNA [NM_007112]	2.17	1.59E-18	54594	24955								
	THBS4	thrombospondin 4 (THBS4), mRNA [NM_003248]	7.99	2.8E-45	67565	8263	0.31	1.44E-15	6783	21857	2.59	4.00E-07	65044	23708
A<R M	TNFAIP6	tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA [NM_007115]	2.52	3.04E-25	2109	830					2.78	8.97E-39	2084	749

7) Proliferation

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ADAMTS1	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1),	0.16	0	779	4846	2.24	1.74E-25	4604	2051	0.45	1.98E-18	846	1849
			0.22	0	1021	4714	2.39	2.92E-25	5726	2390	0.33	0	719	2196
AvR √ M	AHR	aryl hydrocarbon receptor (AHR), mRNA [NM_001621]	0.34	0	3183	9481	2.24	2.10E-21	10103	4460				
	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3), m	3.06	0	3500	1145	0.24	0	1268	5216				
	ANGPTL7	angiopoietin-like 7 (ANGPTL7), mRNA [NM_021146]	66.93	0	12779	192	0.005	0	1284	274406	0.05	0	11741	233940
	APC2	adenomatosis polyposis coli 2 (APC2), mRNA [NM_005883]	0.26	8.35E-13	174	709	7.02	8.65E-19	741	104				
							3.29	4.20E-45	11283	3425				
	APCDD1	adenomatosis polyposis coli down-regulated 1 (APCDD1), mRNA [NM_153000]	0.08	0	2023	26641	15.19	0	27565	1777				
	AREG	amphiregulin (schwannoma-derived growth factor) (AREG), mRNA [NM_001657]	0.07	0	598	8751	9.51	0	8522	881				
S=R PCR	BMP7	bone morphogenetic protein 7 (osteogenic protein 1) (BMP7), mRNA [NM_001719]	0.31	6.31E-44	638	2062	16.33	0	2205	136	5.36	9.92E-13	552	99
	CD44	CD44 antigen (homing function and Indian blood group system) (CD44), transcript variant 1, mRNA [NM	2.52	2.66E-37	14277	5645	0.29	1.37E-29	5892	20369				
	CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen) (CD86), transcript variant 2, mRNA [NM_006889]	3.30	2.90E-20	5172	1567	0.26	5.94E-14	1320	5417				
	CD9	CD9 antigen (p24) (CD9), mRNA [NM_001769]	2.36	3.17E-42	4833	2044	0.35	0	2124	6096				
	CMIP	c-Maf-inducing protein (CMIP), transcript variant C-mip, mRNA [NM_198390]	0.45	2.80E-45	1052	2363	3.31	3.53E-27	2456	736	4.07	2.43E-41	50177	12410
	CMIP						5.61	0	105445	18435				
	CYR61	cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	0.36	1.57E-24	2411	6778					0.34	7.99E-22	3162	9385
			0.33	2.85E-24	4793	14540					0.23	6.16E-25	5002	21658
			0.37	1.66E-10	12641	34956					0.25	1.29E-13	13257	53481
	DCD	dermcidin (DCD), mRNA [NM_053283]	22.74	0	12859	566					23.48	0	12034	513
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	2.10	2.24E-22	1193	568	0.13	0	628	5007	0.26	0	1125	4354
	EGR1	early growth response 1 (EGR1), mRNA [NM_001964]	0.39	1.00E-33	12036	30703					0.46	3.95E-15	10223	22395
	EML4	echinoderm microtubule associated protein like 4 (EML4), mRNA [NM_019063]	0.32	2.29E-27	2311	7308	2.82	1.66E-23	5363	1867				
			0.38	0	2501	6619	2.64	0	7006	2651				
	EPLIN	epithelial protein lost in neoplasm beta (EPLIN), mRNA [NM_016357]	2.13	2.53E-06	13649	6186								
	EPS8	epidermal growth factor receptor pathway substrate 8 (EPS8), mRNA [NM_004447]	2.44	1.28E-19	4533	1848	0.26	1.37E-31	2022	7828	0.61	3.38E-06	4557	7430
	EREG	epiregulin (EREG), mRNA [NM_001432]	0.12	5.57E-38	150	1261	3.05	1.18E-15	629	205	3.45	7.21E-16	537	155
							5.93	2.27E-14	1257	215				
	FGF18	fibroblast growth factor 18 (FGF18), transcript variant 1, mRNA [NM_003862]	3.05	0.00002	986	348					3.55	4.64E-30	1118	318
	FIGF	c-fos induced growth factor (vascular endothelial growth factor D) (FIGF), mRNA [NM_004469]	0.48	1.84E-22	824	1716								
	FSTL3	follistatin-like 3 (secreted glycoprotein) (FSTL3), mRNA [NM_005860]	0.41	7.45E-08	208	509	2.04	0.00002	531	263				
	GPNMB	glycoprotein (transmembrane) nmb (GPNMB), transcript variant 1, mRNA [NM_001005340]	2.06	6.44E-12	16496	7956					2.67	1.13E-23	15867	5948
AvR √ M,W,N	IGFBP6	insulin-like growth factor binding protein 6 (IGFBP6), mRNA [NM_002178]	3.33	1.69E-25	1118	339	0.09	0	380	4180	0.25	6.62E-35	1172	4684
	IGFBP7	insulin-like growth factor binding protein 7 (IGFBP7), mRNA [NM_001553]	2.42	0	174079	71860					2.65	2.79E-19	153744	57411
	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide) (INHBA), mRNA [NM_002192]	0.2	0	4'082	20'587	4.57	0	22595	4945				
	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3) (LGALS3), mRNA [NM_002306]	2.06	0	22673	10983	0.31	0	11067	35700				
	LHFP	lipoma HMGIC fusion partner (LHFP), mRNA [NM_005780]	5.45	0	60602	11123					4.02	1.05E-40	61702	15117

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	LZTS1	leucine zipper, putative tumor suppressor 1 (LZTS1), mRNA [NM_021020]	2.74	2.09E-38	3604	1317	4.86	9.00E-27	1363	278	11.63	0	3849	331
AvR √ M	MDK	midkine (neurite growth-promoting factor 2) (MDK), transcript variant 1, mRNA [NM_001012334]	0.41	2.85E-34	8098	19914	5.72	0	20422	3560	2.19	0	7458	3399
	MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA [NM_000245]	3.49	0	4439	1270	0.32	0	1270	3907				
	MPHOSPH6	M-phase phosphoprotein 6 (MPHOSPH6), mRNA [NM_005792]	0.43	1.22E-06	322	798	3.24	2.59E-11	681	213				
	MTSS1	metastasis suppressor 1 (MTSS1), mRNA [NM_014751]	3.18	9.86E-18	1068	344	0.06	0	308	4795	0.24	0	1117	4639
	NEDD9	neural precursor cell expressed, developmentally down-regulated 9 (NEDD9), mRNA [NM_006403]	2.24	1.42E-10	692	313	0.45	2.92E-07	350	761				
	NOV	nephroblastoma overexpressed gene (NOV), mRNA [NM_002514]	8.90	0	9069	1018					9.90	0	8761	886
AvR √ M	NRG1	neuregulin 1 (NRG1), transcript variant GGF2, mRNA [NM_013962]	0.30	7.15E-25	418	1435	3.31	4.10E-26	1439	437				
	NRG1		0.33	4.17E-42	662	2015	8.10	0	2935	364				
	NRG1		0.14	0	402	2809								
	PAWR	PRKC, apoptosis, WT1, regulator (PAWR), mRNA [NM_002583]	0.47	5.90E-39	1703	3594	3.20	0	4239	1326				
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide (PDGFRB), mRNA [NM_002609]	2.06	2.73E-19	38724	18703					2.32	5.85E-18	33903	14521
	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid) (PLA2G2A), mRNA [NM_000300]	2.67	0	4906	1835	0.08	0	3708	46224	0.13	0	6986	52284
	PLK2	polo-like kinase 2 (Drosophila) (PLK2), mRNA [NM_006622]	0.37	1.31E-37	5208	13943								
SvR √ M	PRG4	proteoglycan 4 (PRG4), mRNA [NM_005807]	18.71	0	4629	246	0.01	0	482	44080	0.12	0	4826	40787
	RAB18	RAB18, member RAS oncogene family (RAB18), mRNA [NM_021252]	2.75	1.54E-19	121724	44300								
	RAB7L1	RAB7, member RAS oncogene family-like 1 (RAB7L1), mRNA [NM_003929]	0.49	9.43E-12	10499	21494	5.34	0	20566	3837	2.81	2.53E-26	11410	3977
	RAB31	RAB31, member RAS oncogene family (RAB31), mRNA [NM_006868]	2.02	3.78E-21	9459	4676	2.31	0	3893	1686	5.23	0	8902	1695
							2.72	0	4903	1801	3.71	0	7026	1895
	REC8L1	REC8-like 1 (yeast) (REC8L1), mRNA [NM_005132]	0.47	4.40E-17	771	1631	5.42	3.17E-13	1900	336	2.98	1.72E-18	765	256
	RGS1	regulator of G-protein signalling 1 (RGS1), mRNA [NM_002922]	0.19	0	644	3415	2.39	6.25E-41	3591	1503	0.47	8.97E-20	620	1340
	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog) (S	3.09	1.14E-33	29119	9360					2.32	3.91E-11	29080	12255
	SDC1	syndecan 1 (SDC1), transcript variant 1, mRNA [NM_001006946]	0.34	3.75E-34	2339	6979	9.81	0	6976	700	4.68	3.17E-24	2507	533
	SPARCL1	SPARC-like 1 (mast9, hevin) (SPARCL1), mRNA [NM_004684]	2.46	1.35E-34	153223	62080								
SvR √ M, PCR	TNC	tenascin C (hexabrachion) (TNC), mRNA [NM_002160]	5.24	0	40057	7601	2.48	0	5955	2403	8.99	0	41901	4677
A<R M	TNFAIP6	tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA [NM_007115]	2.52	3.04E-25	2109	830					2.78	8.97E-39	2084	749
	TNMD	tenomodulin (TNMD), mRNA [NM_022144]	24.00	0	5597	233	0.07	0	156	2131	2.32	2.71E-29	4991	2142
	TNXB	tenascin XB (TNXB), transcript variant XB, mRNA [NM_019105]	5.99	0	22076	3653	0.13	0	3271	25201				
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	0.39	1.28E-32	802	2046	5.48	0	2197	401	2.21	1.07E-09	649	289
	WFDC1	WAP four-disulfide core domain 1 (WFDC1), mRNA [NM_021197]	0.41	4.08E-20	720	1769	2.60	1.04E-13	1764	682				

8) Cytoskeleton

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
S>R IHC,ISH,W,M	ACTA2	actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA [NM_001613]	0.48	0	8420	17567	2.24	0	19617	8719				
AvR √ M	ACTG2	actin, gamma 2, smooth muscle, enteric (ACTG2), mRNA [NM_001615]	0.06	0	1556	25717	9.96	0	26552	2637	0.47	6.91E-12	840	1821
	ACTN2	actinin, alpha 2 (ACTN2), mRNA [NM_001103]	0.06	0	64	1129	16.32	1.67E-42	1217	74				
	ANK3	ankyrin 3, node of Ranvier (ankyrin G) (ANK3), transcript variant 1, mRNA [NM_020987]	2.71	3.07E-18	1044	380	0.41	4.40E-21	411	1006				
	APC2	adenomatosis polyposis coli 2 (APC2), mRNA [NM_005883]	0.26	8.35E-13	174	709	7.02	8.65E-19	741	104				
							3.29	4.20E-45	11283	3425				
	BDKRB1	bradykinin receptor B1 (BDKRB1), mRNA [NM_000710]	0.35	7.22E-14	214	607	3.94	1.35E-10	591	152				
	BDKRB2	bradykinin receptor B2 (BDKRB2), mRNA [NM_000623]	0.48	3.83E-32	2138	4493	2.29	0	4322	1888				
	CGNL1	cingulin-like 1 (CGNL1), mRNA [NM_032866]	2.14	6.80E-30	1888	884	0.23	3.40E-38	970	4222	0.48	2.23E-19	1766	3688
	CMIP	c-Maf-inducing protein (CMIP), transcript variant C-mip, mRNA [NM_198390]	0.45	2.80E-45	1052	2363	3.31	3.53E-27	2456	736	4.07	2.43E-41	50177	12410
	CMIP						5.61	0	105445	18435				
	DST	dystonin (DST), transcript variant 1eA, mRNA [NM_015548]	2.03	2.43E-13	1029	502					5.29	6.99E-20	642	123
			2.10	1.30E-17	4993	2375					2.91	4.13E-10	1031	338
											2.10	3.96E-12	4612	2188
	DTNA	dystrobrevin, alpha, mRNA (cDNA clone MGC:12368 IMAGE:3933795), complete cds. [BC005300]	3.10	0.10767	823	230								
	EML4	echinoderm microtubule associated protein like 4 (EML4), mRNA [NM_019063]	0.32	2.29E-27	2311	7308	2.82	1.66E-23	5363	1867				
			0.38	0	2501	6619	2.64	0	7006	2651				
	EPLIN	epithelial protein lost in neoplasm beta (EPLIN), mRNA [NM_016357]	2.13	2.53E-06	13649	6186								
	FGF18	fibroblast growth factor 18 (FGF18), transcript variant 1, mRNA [NM_003862]	3.05	0.00002	986	348					3.55	4.64E-30	1118	318
	FSD1	fibronectin type III and SPRY domain containing 1 (FSD1), mRNA [NM_024333]	0.49	1.24E-27	5712	11598	10.16	0	12113	1185	5.82	0	4401	753
	ITGA8	integrin, alpha 8 (ITGA8), mRNA [NM_003638]	3.84	7.06E-41	2259	590					6.47	0	2527	391
	ITGA9	integrin, alpha 9 (ITGA9), mRNA [NM_002207]	2.42	3.60E-12	1010	406					3.57	6.80E-13	921	247
	KIF13B	kinesin family member 13B (KIF13B), mRNA [NM_015254]	2.40	1.43E-31	12112	5041								
	KIF5C	mRNA for KIAA0531 protein, partial cds. [AB011103]	3.79	6.90E-20	1458	373					10.77	1.60E-18	1560	136
	MGC8685	tubulin, beta polypeptide paralog (RP11-506K6.1), mRNA [NM_178012]	3.03	0	7027	2317	5.97	0	2153	361	12.51	0	7336	587
	MYBPC1	myosin binding protein C, slow type (MYBPC1), transcript variant 2, mRNA [NM_206819]	0.41	0.00109	240	673					0.23	4.43E-15	221	988
	MYBPH	myosin binding protein H (MYBPH), mRNA [NM_004997]	0.42	2.26E-18	679	1637								
	MYL1	myosin, light polypeptide 1, alkali; skeletal, fast (MYL1), transcript variant 3f, mRNA [NM_079422]	0.12	1.24E-29	118	1031	4.51	3.12E-19	1018	229				
	MYLK	myosin, light polypeptide kinase (MYLK), transcript variant 1, mRNA [NM_053025]	0.50	5.35E-43	2955	5636	2.35	1.52E-20	5877	2481				
							2.03	3.58E-11	24837	12243				
	MYO10	myosin X (MYO10), mRNA [NM_012334]	0.47	8.67E-42	1068	2284								
	MYO5A	myosin VA (heavy polypeptide 12, myoxin) (MYO5A), mRNA [NM_000259]	2.21	2.34E-18	3233	1456								
	MYOC	myocilin, trabecular meshwork inducible glucocorticoid response (MYOC), mRNA [NM_000261]	10.52	0	3158	300	0.02	0	786	49667	0.07	0	3387	51303
	NEB	nebulin (NEB), mRNA [NM_004543]	0.21	0	384	1805	3.76	1.01E-34	1731	462				
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide (PDGFRB), mRNA [NM_002609]	2.06	2.73E-19	38724	18703					2.32	5.85E-18	33903	14521

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	PPP1R12B	protein phosphatase 1, regulatory (inhibitor) subunit 12B (PPP1R12B), transcript variant 2, mRNA [NM_	0.44	9.97E-12	5587	12707								
	TNNT1	troponin T1, skeletal, slow, mRNA (cDNA clone IMAGE:3531880), partial cds. [BC022086]	0.17	0	318	1876	5.81	5.92E-15	1930	331				
	TTN	titin (TTN), transcript variant novex-3, mRNA [NM_133379]	0.11	0	633	5824	11.65	0	5738	492				
	VAV3	vav 3 oncogene (VAV3), mRNA [NM_006113]	0.39	1.28E-32	802	2046	5.48	0	2197	401	2.21	1.07E-09	649	289

9) Complement and coagulation cascades

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3), m	3.06	0	3500	1145	0.24	0	1268	5216				
	BDKRB1	bradykinin receptor B1 (BDKRB1), mRNA [NM_000710]	0.35	7.22E-14	214	607	3.94	1.35E-10	591	152				
	BDKRB2	bradykinin receptor B2 (BDKRB2), mRNA [NM_000623]	0.48	3.83E-32	2138	4493	2.29	0	4322	1888				
	BF	B-factor, properdin (BF), mRNA [NM_001710]	3.08	5.41E-22	1658	528	0.30	5.87E-33	594	1973				
	C2	complement component 2 (C2), mRNA [NM_000063]	2.46	1.15E-33	16874	6818								
	CD44	CD44 antigen (homing function and Indian blood group system) (CD44), transcript variant 1, mRNA [NM	2.52	2.66E-37	14277	5645	0.29	1.37E-29	5892	20369				
	CFH	complement factor H (CFH), transcript variant 1, mRNA [NM_000186]	2.07	0.0007	1015	522	0.30	7.71E-10	563	1957	0.46	3.53E-11	1027	2260
	DF	D component of complement (adipsin) (DF), mRNA [NM_001928]	5.98	0	7807	1306	0.03	0	1567	46254	0.18	0	8228	45963
	EDN1	endothelin 1 (EDN1), mRNA [NM_001955]	2.10	2.24E-22	1193	568	0.13	0	628	5007	0.26	0	1125	4354
	F2RL2	coagulation factor II (thrombin) receptor-like 2 (F2RL2), mRNA [NM_004101]	0.26	0	962	3654	15.96	0	3448	213	5.05	1.89E-20	1023	196
	F3	coagulation factor III (thromboplastin, tissue factor) (F3), mRNA [NM_001993]	0.27	3.52E-23	303	1161					0.32	1.49E-09	218	659
	FGA	fibrinogen, A alpha polypeptide (FGA), transcript variant alpha-E, mRNA [NM_000508]	0.03	2.51E-15	94	1879								
	HRH1	histamine receptor H1 (HRH1), mRNA [NM_000861]	2.15	1.62E-12	1587	744								
			2.08	6.10E-09	3428	1640								
	PLAT	plasminogen activator, tissue (PLAT), transcript variant 1, mRNA [NM_000930]	0.16	0	2024	12582	2.01	2.33E-08	13121	6523	0.33	6.95E-27	1738	5366
	PLAU	plasminogen activator, urokinase (PLAU), mRNA [NM_002658]	2.50	1.11E-30	18274	7260					4.10	0	17120	4155
	PLCB2	phospholipase C, beta 2 (PLCB2), mRNA [NM_004573]	2.49	6.98E-39	2585	1038	2.40	0	30170	12586				
	PTGFR	prostaglandin F receptor (FP) (PTGFR), mRNA [NM_000959]	2.30	3.57E-28	1334	580	0.20	2.80E-45	585	2951				
AvR √ M	PTGIS	prostaglandin I2 (prostacyclin) synthase (PTGIS), mRNA [NM_000961]	4.84	0	33151	6851	0.40	8.8E-32	5830	14554				
AvR √ IHC,N,W	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), m	0.25	7.43E-15	150	606	2.83	1.27E-18	713	252				
	SELE	selectin E (endothelial adhesion molecule 1) (SELE), mRNA [NM_000450]	2.34	9.56E-10	903	397	0.37	1.17E-09	414	1132				
	SELP	selectin P (granule membrane protein 140kDa, antigen CD62) (SELP), mRNA [NM_003005]	2.34	9.80E-14	910	394	0.47	1.79E-09	353	744				
	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5 (SERPI	4.02	0.04679	659	147	4.73	4.20E-16	3499	728	2.80	9.34E-08	1838	668

10) Others

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	ARMC7	armadillo repeat containing 7 (ARMC7), mRNA [NM_024585]	0.45	5.21E-32	5836	13129	5.82	1.97E-35	11705	1990	3.00	1.35E-23	3413	1095
	CDO1	cysteine dioxygenase, type I (CDO1), mRNA [NM_001801]	3.49	1.76E-34	1743	502	0.03	0	777	30290	0.07	0	1715	24644
	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta (CEBPD), mRNA [NM_005195]	0.40	3.01E-34	7233	18105	0.15	0	18519	125697	0.06	0	9484	148572
	CRISP2	cysteine-rich secretory protein 2 (CRISP2), mRNA [NM_003296]	0.42	6.68E-15	5332	12672	15.55	0	14461	936	7.65	1.14E-38	3562	445
	CMYA3	cardiomyopathy associated 3, mRNA (cDNA clone IMAGE:4338489), complete cds. [BC022888]	0.11	3.67E-42	195	1687								
			0.23	4.37E-07	87	408								
AvR √ M	CRIP1	cysteine-rich protein 1 (intestinal) (CRIP1), mRNA [NM_001311]	4.89	4.94E-17	2471	506	0.31	6.83E-35	488	1556				
	DMRT2	doublesex and mab-3 related transcription factor 2 (DMRT2), mRNA [NM_006557]	12.29	0	1886	154					8.02	0	1688	211
	EBF	early B-cell factor (EBF), mRNA [NM_024007]	2.48	6.72E-07	503	196	0.22	1.13E-22	242	1101	0.44	3.40E-17	545	1217
	FCN2	ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin) (FCN2), transcript variant SV0, mRNA [N	0.48	7.24E-10	504	1064	5.26	5.00E-12	1144	222	3.48	3.79E-08	651	202
	G1P2	interferon, alpha-inducible protein (clone IFI-15K) (G1P2), mRNA [NM_005101]	0.46	2.17E-23	59237	128260	11.97	0	129497	10868	7.08	0	55435	7835
	GPNMB	glycoprotein (transmembrane) nmb (GPNMB), transcript variant 1, mRNA [NM_001005340]	2.06	6.44E-12	16496	7956					2.67	1.13E-23	15867	5948
	GPR32	G protein-coupled receptor 32 (GPR32), mRNA [NM_001506]	0.42	3.87E-26	25679	61517	8.36	0	61762	7388	3.68	0	22196	6022
	GPR44	G protein-coupled receptor 44 (GPR44), mRNA [NM_004778]	0.38	9.49E-14	438	1184	4.52	1.09E-12	1207	273	2.69	6.88E-07	669	267
	HEXA	HEXA {HEXA4bpDeltaA mutation, exon 11} [human, Tay-Sachs disease patient, mRNA Partial Mutant, 7	0.46	3.92E-13	22564	49195	11.62	0	48620	4175	6.04	0	19539	3225
							3.51	0	3599	1025				
	HMGN4	high mobility group nucleosomal binding domain 4 (HMGN4), mRNA [NM_006353]	0.49	0	40423	83263	7.68	0	75900	10029	4.67	0	32987	7089
	HOMER1	homer homolog 1 (Drosophila) (HOMER1), mRNA [NM_004272]	0.43	1.95E-25	630	1461	8.18	1.18E-40	1471	178	3.75	5.80E-10	555	142
	IGHG3	Human Ig gamma3 heavy chain disease OMM protein mRNA. [J00231]	0.42	1.19E-35	1026	2435	4.11	0	2541	619				
	ITM2B	integral membrane protein 2B (ITM2B), mRNA [NM_021999]	2.07	1.55E-38	29225	14116								
	KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1 (KCNMB1), mRN	0.38	0	1256	3292	2.82	3.14E-38	3270	1156				
	LRRN1	leucine rich repeat neuronal 1 (LRRN1), mRNA [NM_020873]	0.36	0	3754	10566	26.52	3.92E-44	8611	313	12.28	0	3647	297
	NFIB	nuclear factor I/B (NFIB), mRNA [NM_005596]	2.13	1.33E-07	1123	544	0.41	6.77E-11	568	1391				
	NFIX	nuclear factor I/X (CCAAT-binding transcription factor) (NFIX), mRNA [NM_002501]	2.84	2.18E-26	11767	4098								
	NR2F1	nuclear receptor subfamily 2, group F, member 1 (NR2F1), mRNA [NM_005654]	0.15	0	479	3120	8.84	0	3649	413				
	NR4A1	nuclear receptor subfamily 4, group A, member 1 (NR4A1), transcript variant 1, mRNA [NM_002135]	0.22	0	5225	23757					0.20	0	5256	26534
	NR4A2	nuclear receptor subfamily 4, group A, member 2 (NR4A2), transcript variant 1, mRNA [NM_006186]	0.30	0	810	2678								
	NUMA1	nuclear mitotic apparatus protein 1 (NUMA1), mRNA [NM_006185]	2.04	3.99E-13	2485	1223	0.34	4.87E-32	1279	3755				
	PTPNS1	protein tyrosine phosphatase, non-receptor type substrate 1 (PTPNS1), mRNA [NM_080792]	2.04	5.20E-09	2889	1413	0.33	1.68E-22	1479	4567				
	RAB11FIP1	RAB11 family interacting protein 1 (class I) (RAB11FIP1), transcript variant 2, mRNA [NM_001002233]	0.40	3.79E-31	1872	4650	9.03	0	4919	545	3.62	1.63E-11	1316	341
	RARB	Human retinoic acid receptor-beta associated open reading frame, complete sequence. [M62303]	0.13	1.82E-08	50	451								
	REC8L1	REC8-like 1 (yeast) (REC8L1), mRNA [NM_005132]	0.47	4.40E-17	771	1631	5.42	3.17E-13	1900	336	2.98	1.72E-18	765	256
	RPA4	replication protein A4, 34kDa (RPA4), mRNA [NM_013347]	2.21	5.62E-19	1322	593	0.33	5.08E-40	817	2457				
	TADA3L	transcriptional adaptor 3 (NGG1 homolog, yeast)-like (TADA3L), transcript variant 1, mRNA [NM_00635	0.48	9.81E-44	3412	7108	6.12	2.35E-39	7142	1152	3.31	3.12E-30	2703	806
S<R M	TFPI2	tissue factor pathway inhibitor 2 (TFPI2), mRNA [NM_006528]	0.39	9.07E-17	362	935	2.02	1.14E-16	1000	494				
	TPST1	tyrosylprotein sulfotransferase 1 (TPST1), mRNA [NM_003596]	0.47	2.95E-38	3601	7677	3.21	2.14E-22	8549	2620				

			superficial / aggressive				aggressive / reference				superficial / reference			
			ratio	pvalue	superfic	aggr	ratio	pvalue	aggr	ref	ratio	pvalue	superfic	ref
	TREM1	triggering receptor expressed on myeloid cells 1 (TREM1), mRNA [NM_018643]	2.36	5.49E-15	874	375	0.45	6.05E-09	321	710				
	TSPAN2	tetraspanin 2 (TSPAN2), mRNA [NM_005725]	2.31	6.48E-12	1139	503					4.40	2.54E-36	1049	240
	UBOX5	U-box domain containing 5 (UBOX5), transcript variant 1, mRNA [NM_014948]	0.41	9.01E-12	241337	588394	0.31	1.03E-16	241909	791541	0.34	6.18E-15	281568	826316
	ZNF365	mRNA for zinc finger protein (ZNF365A gene). [AJ505147]	4.16	1.68E-16	651	160								

Gene list C: Gene expression studies published in the literature that could be confirmed by our own data

in Gene lists A or B

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
ACTG2	Actin, gamma 2, smooth muscle, enteric	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Ref, Super < Aggr
ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	Shih *et al.,* 2009	Super	tissue samples and cell cultures: Super > control (RT-PCR)	Super > Ref	Aggr > Ref, Super > Ref
AHR	Aryl hydrocarbon receptor	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr
AKR1C1	Aldo-keto reductase family 1, member C1	Pan *et al.,* 2003	Super	frozen tissues: 6 Super palmar fascia vs 2 Ref (normal palmar fascia): Super < Ref (Atlas microarray)	Super < Ref	Aggr < Ref, Super < Ref, Super > Aggr
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	Pan *et al.,* 2003	Super	frozen tissues: 6 Super palmar fascia vs 2 Ref (normal palmar fascia): Super < Ref (Atlas microarray)	Super < Ref	Aggr < Ref, Super < Ref
ANGPTL2	Angiopoietin-like 2	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super > Aggr
APP	Amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	Pan *et al.,* 2003; Qian *et al*., 2004	Super	Pan *et al.*, 2003: frozen tissues: 6 Super palmar fascia vs 2 Ref (normal palmar fascia): Super > Ref (Atlas microarray) Qian *et al*., 2004: frozent tissues: 9 Super palmar fasica vs adjacent normal tendon: Super > Ref (Atlas microarray)	Super > Ref	Aggr > Ref, Super > Ref
ARL4C	ADP-ribosylation factor-like 4C (ARL7)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
ASPN	Asporin (LRR class 1)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super > Aggr
BMP1	Bone morphogenetic protein 1	Shin *et al.,* 2004	Super	cell culture: nodule and cord Super fibroblasts vs normal palmar fascia fibroblasts: Super = Ref (RT-PCR)	Super = Ref	Aggr > Ref
BMP10	Bone morphogenetic protein 10	Shin *et al.,* 2004	Super	cell culture: nodule and cord Super fibroblasts vs normal palmar fascia fibroblasts: Super = Ref (no expression) (RT-PCR)	Super = Ref	not differentially expressed
BMP2	Bone morphogenetic protein 2	Shin *et al.,* 2004	Super	cell culture: nodule and cord Super fibroblasts vs normal palmar fascia fibroblasts: Super = Ref (no expression) (RT-PCR)	Super = Ref	not differentially expressed
BMP3	Bone morphogenetic protein 3	Shin *et al.,* 2004	Super	cell culture: nodule and cord Super fibroblasts vs normal palmar fascia fibroblasts: Super = Ref (no expression) (RT-PCR)	Super = Ref	not differentially expressed
BMP5	Bone morphogenetic protein 5	Shin *et al.,* 2004	Super	cell culture: nodule and cord Super fibroblasts vs normal palmar fascia fibroblasts: Super = Ref (no expression) (RT-PCR)	Super = Ref	not differentially expressed
C1QTNF3	C1q and tumor necrosis factor related protein 3	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
CACNA2D3	Calcium channel, voltage-dependent, alpha 2/delta 3 subunit	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
CALB2	Calbindin 2, 29kDa (calretinin)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr
CCND1	Cyclin D1	Saito *et al.*, 2002; Saito *et al.*, 2001	Aggr	Saito *et al*., 2002: frozen samples (12 Aggrs) Aggr > skeletal muscle (real time RT-PCR), 3 Aggrs with beta-catenin mutated > unmutated (real time RT-PCR) Saito *et al.*, 2001: paraffin blocks (38 Aggrs) Association between nuclear beta-cat and CCND1 expr. (IHC)	Aggr > Ref	Aggr > Ref, Super > Ref
CCND2	Cyclin D2	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
CHN1	Chimerin (chimaerin) 1 (GTPase-activating protein, rho, 2, RHOGAP2)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fascia: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref
CLEC11A	C-type lectin domain family 11, member A	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref
CLEC2B	C-type lectin domain family 2, member B (CLECSF2)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr
CLEC3B	C-type lectin domain family 3, member B (tetranectin TNA (plasminogen binding protein))	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super > Aggr
CNN1	Calponin 1, basic, smooth muscle	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr
COL1	Collagen, type I	Zamora *et al.*, 1994; Kopp *et al.*, 2006	Super	Zamora *et al*., 1994: paraffin blocks: 36 nodular Super vs 24 cord Super: nodular > cord (IHC) Kopp *et al*., 2006: cell culture: cord Super fibroblasts vs. control tissue fibroblasts: Super > Ref (Western)	Super > Ref	Aggr > Ref, Super > Ref
COL11A1	Collagen, type XI, alpha 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr
COL12A1	Collagen, type XII, alpha 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref
COL14A1	Collagen, type XIV, alpha 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL1A1	Collagen, type I, alpha 1	Naito *et al*., 1998; Skubitz and Skubitz, 2004	Aggr	Naito *et al*., 1998: cell culture: 6 Aggrs vs. human skin fibroblasts (HSF): Aggr = HSF (real time RT-PCR) Skubitz and Skubitz, 2004: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL2A	Collagen, type II, alpha	Qian *et al*., 2004	Super	frozent tissues: 9 Super palmar fasica vs adjacent normal tendon: Super > Ref (Atlas microarray)	Super > Ref	Super > Ref
COL3	Collagen, type III	Howard *et al*., 2003	Super	old reference: cord Super > normal palmar fascia	Super > Ref	Aggr > Ref, Super > Ref, Super < Aggr (COL3A1)
COL3A1	Collagen, type III, alpha 1	Naito *et al*., 1998; Skubitz and Skubitz, 2004	Aggr	Naito *et al*., 1998: cell culture: 6 Aggrs vs. human skin fibroblasts (HSF): Aggr > HSF (real time RT-PCR) Skubitz and Skubitz, 2004: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr

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COL4	Collagen, type IV	Berndt *et al*., 1994; Kosmehl *et al.*, 1995; Magro *et al.*, 1997	Super	Berndt *et al*., 1994: 13 nodular palmar Super: expression restricted to active proliferating nodules (ACTA2+) compared to nearby palmar aponeurosis (IHC) Kosmehl *et al*., 1995: nodular palmar Super (? cases): proliferative noduli > surrounding aponeurosis (IHC) Magro *et al*., 1997: 24 nodular palmar Super: expression restricted to proliferative phase compared to involutional and residual phase (IHC)	Super > Ref	Aggr > Ref, Super > Ref (COL4A1)
COL5A1	Collagen, type V, alpha 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr
COL5A2	Collagen, type V, alpha 2	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL5A2	Collagen, type V, alpha 2	Lee *et al.*, 2006; Zhang *et al*., 2008	Super	Lee *et al*., 2006: frozen tissues: cord Super (4) vs. Super-adjacent control fascia (4) vs. normal palmar fascia (3): Super > Ref (cDNA Array); Zhang *et al*., 2008: Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
COL6	Collagen, type VI	Thurston, 2003; Magro *et al.*, 1995	Super	Magro *et al*., 1995: 26 palmar/plantar Super: proliferative phase > involutional, residual phase and normal aponeurosis (IHC)	Super > Ref	Aggr > Ref, Super > Ref (COL6A1,2,3)
COL6A1	Collagen, type VI, alpha 1	Skubitz and Skubitz, 2004; Denys *et al*., 2004b	Aggr	Skubitz and Skubitz, 2004: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array) Denys *et al*., 2004b : cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL6A2	Collagen, type VI, alpha 2	Skubitz and Skubitz, 2004; Denys *et al*., 2004b	Aggr	Skubitz and Skubitz, 2004: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array) Denys et al., 2004b: cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL6A3	Collagen, type VI, alpha 3	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
COL6A3	Collagen, type VI, alpha 3	Kraljevic Pavelic *et al*., 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Aggr > Ref, Super > Ref
COL8A1	Collagen, type VIII, alpha 1	Lee *et al.*, 2006; Zhang *et al*., 2008	Super	Lee *et al.*, 2006: frozen tissues: cord Super (4) vs. Super-adjacent control fascia (4) vs. normal palmar fascia (3): Super > Ref (cDNA Array); Zhang *et al*., 2008: Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
CRIP1	Cysteine-rich protein 1 (intestinal)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super > Aggr
CRLF1	Cytokine receptor-like factor 1	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super < Ref
CSRP2	Cysteine and glycine-rich protein 2	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref

Gene		Paper	Literature	Paper		Own results
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CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Tejpar *et al*., 1999; Saito *et al*., 2002; Montgomery *et al*., 2001; Carlson *et al*., 2007; Alman *et al*., 1997b; Ferenc *et al*., 2009	Aggr	Tejpar *et al*., 1999: paraffin blocks (42 Aggrs) Aggr (cytopl, nuc) > normal marginal facial tissue (IHC) Saito *et al*., 2002: frozen samples (12 Aggrs) Aggr > skeletal muscle (real time RT-PCR), 3 Aggrs with beta-catenin mutated > unmutated (real time RT-PCR), frozen samples (12 Aggrs): nucl. accumulation in all samples (IHC) Montgomery *et al*., 2001: paraffin blocks 5 Aggrs: nuclear staining in all five cases tested (IHC) Carlson *et al*., 2007: paraffin blocks: nuclear accumulation Aggr > several other spindle cell lesions (IHC) Alman *et al*., 1997b: six cases of sporadic aggressive fibromatosis: higher level of β-catenin protein than surrounding normal tissue Ferenc *et al*., 2009: 35 cases of aggressive fibromatoses: nuclear and cytoplasmic β-catenin staining	Aggr > Ref	Aggr > Ref
CTSK	Cathepsin K (pycnodysostosis)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
CYB5	Cytochrome b-5	Pan et al., 2003	Super	frozen tissues: 6 Super palmar fascia vs 2 Ref (normal palmar fascia): Super < Ref (Atlas microarray)	Super < Ref	Super < Ref
DCN	Decorin (bone proteoglycan II; dermatan sulphate proteoglycans II)	Kozma *et al*., 2005, Qian *et al*., 2004	Super	Kozma *et al*., 2005: frozen tissues: 15 Super fascia vs 14 normal palmar fasica: DS chain molecular mass increase, iduronate disaccarid clusters increase, oversulfatation Qian *et al*., 2004: frozent tissues: 9 Super palmar fasica vs adjacent normal tendon: Super < Ref (Atlas microarray)	Super < Ref	Aggr < Ref, Super < Ref
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super > Aggr
EGFL3	EGF-like-domain, multiple 3	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
ELN	Elastin	Naito et al., 1998	Aggr	cell culture: 6 Aggrs vs. human skin fibroblasts (HSF): Aggr > HSF (real time RT-PCR)	Aggr > Ref	Aggr > Ref, Super > Ref
EPHB3	Ephrin receptor EphB3	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
FAP	Fibroblast activation protein, alpha	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
FAP	Fibroblast activation protein, alpha	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
FN	Fibronectin	Zamora *et al*., 1994; Tomasek *et al*., 1986; Halliday *et al*., 1994; Magro *et al*., 1995b, Kosmehl *et al*., 1995; Berndt *et al*., 1995, Magro *et al*., 1997; Howard *et al*., 2004; Lee *et al*., 2006	Super	Zamora *et al*., 1994: paraffin blocks: 36 nodular superfic vs 24 cord superfic: nodular > cord (IHC) Tomasek *et al*., 1986: nodular palmar superfic (? cases) vs normal palmar fascia fibroblasts: Superfic > Ref (IHC) Halliday et al., 1994: Superfic (? cases) vs normal palmar fascia: proliferative, involutional phase: A-FN > B-FN; residual phase: weakly A-FN, not B-FN; normal fascia: A-FN, B-FN not present; thus: Superfic > Ref (IHC) Magro *et al*., 1995b: 23 Superfic: proliferative, involutional phase > residual phase (IHC) Kosmehl *et al*., 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC, ISH) Berndt *et al*., 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (ISH, IHC) Magro *et al*., 1997: 24 nodular palmar superfic: expression restricted to proliferative phase compared to involutional and residual phase (IHC) Howard *et al*., 2004: palmar superfic (? cases) tissue and cell culture vs adjacent normal palmar fascia: Superfic > Ref (IHC, western) Lee *et al*., 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. norma	Super > Ref	Aggr > Ref, Super > Ref

Gene		Paper	Literature	Paper		Own results
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HMCN1	Hemicentin 1 (fibulin 6 FIBL-6)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
HOXB2	Homeo box B2	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
IGFBP6	Insulin-like growth factor binding protein 6	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array, Northern, Western)	Aggr < Ref	Aggr < Ref, Super < Ref, Super > Aggr
IGSF4	Immunoglobulin superfamily, member 4	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super > Aggr
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Magro *et al.*, 1995b, Magro *et al.*, 1997; Lee *et al*., 2006	Super	Magro *et al*., 1995b: 23 Superfic: proliferative, involutional phase > residual phase (A5B1) (IHC) Magro *et al*., 1997: 24 nodular palmar superfic: expression restricted to proliferative phase compared to involutional and residual phase (A5B1) (IHC) Lee *et al*., 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array)	Super > Ref	Aggr > Ref, Super > Ref
LAMB1	Laminin, beta 1	Kosmehl *et al*., 1995	Super	nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC)	Super > Ref	Aggr > Ref, Super > Ref
LGALS1	Galectin-1	Kraljevic Pavelic et al., 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Aggr > Ref, Super > Ref
LIPE	Lipase, hormone-sensitive	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	Aggr < Ref, Super < Ref
LRRC17	Leucine rich repeat containing 17 (LRRC17), mRNA [NM_005824]	Lee et al., 2006; Zhang et al., 2008	Super	Lee *et al*., 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array); Zhang *et al*., 2008: Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) (MAFB), mRNA [NM_005461]	Lee et al., 2006; Zhang et al., 2008	Super	70: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array); 74: Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
MDK	Midkine (neurite growth-promoting factor 2)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr
MMP1	Matrix metalloproteinase 1	Denys *et al*., 2004a	Aggr	cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR)	Aggr > Ref	Aggr > Ref, Super < Aggr
MMP11	Matrix metalloproteinase 11	Denys *et al*., 2004a; Kong *et al.*, 2004	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR) Kong *et al*., 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr = Ref (real time RT-PCR)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr
MMP14	Matrix metalloproteinase 14	Denys *et al*., 2004a; Kong *et al.*, 2004	Aggr	Denys *et al*., 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr = Ref (real time RT-PCR) Kong *et al.*, 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr = Ref (real time RT-PCR)	Aggr = Ref	Super > Ref
MMP14	Matrix metalloproteinase 14	Johnston *et al*., 2007	Super	frozen tissues: nodule superfic (? samples) vs normal palmar fascia (?): Superfic > Ref (realtime RT-PCR)	Super > Ref	Super > Ref

Gene		Paper	Literature	Paper		Own results
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MMP2	Matrix metalloproteinase 2	Ulrich *et al.*, 2003; Qian *et al.,* 2004	Super	Ulrich *et al.*, 2003: Sera and tissue samples of 22 superfic vs sera and palmar fascia of 20 healthy controls: Superfic = Ref (ELISA for sera, IHC for tissue samples) Qian *et al*., 2004 : frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	Aggr > Ref, Super > Ref
MMP9	Matrix metalloproteinase 9	Denys *et al*., 2004a; Kong *et al.*, 2004	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr = Ref (real time RT-PCR) Kong *et al.,* 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr = Ref (real time RT-PCR)	Aggr = Ref	not differentially expressed
MMP9	Matrix metalloproteinase 9	Ulrich *et al*., 2003	Super	Sera and tissue samples of 22 superfic vs sera and palmar fascia of 20 healthy controls: Superfic = Ref (ELISA for sera, IHC for tissue samples)	Super = Ref	not differentially expressed
NEFH	Neurofilament, heavy polypeptide 200kDa	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
NPTX2	Neuronal pentraxin II	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
NRG1	Neuregulin 1	Skubitz and Skubitz, 2004; Denys *et al.*, 2004b	Aggr	Skubitz and Skubitz, 2004: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array) Denys *et al.*, 2004b : cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr
POSTN	periostin, osteoblast specific factor 2 (OSF2)	Qian *et al*., 2004, Vi *et al.*, 2009; Shih *et al.*, 2009	Super	Qian *et al.*, 2004: frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray) Vi *et al*., 2009: IHC: diseased cord > normal palmar fascia Shih *et al.*, 2009: tissue samples and cell cultures: Super > control (RT-PCR)	Super > Ref	Aggr > Ref, Super > Ref
PRG4	Proteoglycan 4	Satish *et al.*, 2008	Super	cell cultures superficial fibromatosis vs. normal fibroblasts (2 microarray platforms)	Super < Ref	Aggr < Ref, Super < Ref, Super > Aggr
PRSS11	HtrA serine peptidase 1 (HTRA1); IGFBP5 protease; protease, serine, 11 (IGF binding)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
PTGIS	Prostaglandin I2 (prostacyclin) synthase	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super > Ref, Super > Aggr
PTGS2	Prostaglandin-endoperoxide synthase 2 = cyclooxygenase 2b (COX-2)	Poon *et al*., 2001; Sharma *et al*., 2003	Aggr	Poon *et al*., 2001: frozen samples (6 Aggrs): Aggr > Fasica (Northern, Western) Sharma *et al.*, 2003: case report: Aggr < adjoining tissue (IHC)	Aggr > Ref	Aggr > Ref, Super < Aggr
PTN	Pleiotrophin (OSF1, heparin binding growth factor 8, neurite growth-promoting factor 1)	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	Aggr > Ref, Super > Ref
PTX3	Pentraxin-related gene, rapidly induced by IL-1 beta (TNFAIP5)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super < Ref
RAB31	Member 31 RAS oncogene family	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
SALL4	Sal-like 4 (Drosophila)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
SC65	Synaptonemal complex protein SC65	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref

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SERPINE1	serpin peptidase inhibitor, clade E, member 1 (plasminogen activator inhibitor type 1, PAI-1)	Kopp *et al.*, 2006	Super	cell culture: cord superfic fibroblasts vs. control tissue fibroblasts: Superfic = Ref (Western)	Super = Ref	not differentially expressed
SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	Howard *et al*., 2004; Lee *et al.*, 2006	Super	Howard *et al.*, 2004: palmar superfic (? cases) tissue and cell culture vs adjacent normal palmar fascia: Superfic > Ref (western) Lee *et al.*, 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array)	Super > Ref	Aggr > Ref, Super > Ref
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
SPON2	Spondin 2, extracellular matrix protein	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super > Aggr
TCF7	T cell specific transcription factor 7 (TCF1)	Tejpar *et al*., 2001	Aggr	frozen samples (10 Aggrs): Aggr = Ref (10 Fascia same patients) (not expressed !) (RT-PCR)	Aggr = Ref	not differentially expressed
TCF7L1	T cell specific transcription factor 7-like 1 (TCF3)	Tejpar *et al*., 2001	Aggr	frozen samples (10 Aggrs): Aggr = Ref (10 Fascia same patients) (all 10 expressed it !) (RT-PCR)	Aggr = Ref	not differentially expressed
TCF7L2	T cell specific transcription factor 7-like 2 (TCF4)	Tejpar *et al*., 2001	Aggr	frozen samples (10 Aggrs): Aggr (3/10) > Ref (10 Fascia same patients) (0/10) (RT-PCR)	Aggr = Ref	Super < Ref, Super < Aggr
TGFB2	Transforming growth factor beta 2	Badalamente *et al.*, 1996; Kuhn *et al.*, 2001; Kuhn *et al.,* 2002; Berndt *et al.*, 1995; Lee *et al.*, 2006; Zhang *et al.*, 2008	Super	Badalamente *et al.*, 1996: Superfic (? cases): high intracellular expression in myofibroblasts in the proliferative and involutional stages. Fibroblasts in the residual stage did not contain TGFB2. It is not present in the ECM (IHC). Cell cultures with superfic myofibroblasts: addition of TGFB2: proliferation increases, but TGFB2 > TGFB1. Kuhn *et al.*, 2001: human superfic explants and transfered in rat. In rat: addition of TGFB2: increase of COL1 and COL3. Cell cultures: treatemtent with TGFB2: increase of DNA synthesis, protein production and fibroblast kinetics. Kuhn *et al.*, 2002: cell cultures: superfic vs normal carpal tunnel affected fascia: Superfic > Ref (ELISA) Berndt *et al.*, 1995: nodular palmar superfic (? cases): expression exclusively in proliferative noduli, not in surrounding aponeurosis (IHC) Lee *et al.,* 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array) Zhang *et al.*, 2008: cord superfic vs. superfic-adjacent control fascia (cDNA Array)	Super > Ref	Super > Ref
TGFB3	Transforming growth factor beta 3	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
TGFB3	Transforming growth factor beta 3	Berndt *et al*., 1995; Zhang *et al.*, 2008	Super	Berndt *et al.*, 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC) Zhang *et al*., 2008: cord superfic vs. superfic-adjacent control fascia (cDNA Array): not differentially expressed !	Super > Ref	Aggr > Ref, Super > Ref
TGFBI	Transforming growth factor, beta-induced, 68kDa	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Aggr > Ref, Super > Ref
THBS2	Thrombospondin 2	Zhang *et al.*, 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	Aggr > Ref, Super > Ref
TIMP2	Metallopeptidase inhibitor 2 (tissue inhibitor of metalloproteinase 2)	Ulrich *et al.*, 2003	Super	37: Sera and tissue samples of 22 superfic vs sera and palmar fascia of 20 healthy controls: Superfic > Ref (IHC for tissue samples)	Super > Ref	Aggr > Ref, Super > Ref

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
TIMP3	Metallopeptidase inhibitor 3 (tissue inhibitor of metalloproteinase 3)	Denys *et al.,* 2004a	Aggr	cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr < Ref (real time RT-PCR)	Aggr < Ref	Aggr < Ref
TMSB4X	Thymosin, beta 4, X-linked	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	Super > Ref
TNC	Tenascin C	Lee *et al.*, 2006; Shih *et al.*, 2009	Super	Lee *et al.*, 2006: frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array) Shih *et al*., 2009: tissue samples and cell cultures: Super > control (RT-PCR)	Super > Ref	Aggr > Ref, Super > Ref, Super > Aggr
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin OPG)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr < Ref, Super < Ref
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4 (CD134 ligand)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
TP53	Tumor protein p53 (Li-Fraumeni syndrome)	Muller *et al.*, 1996; Sharma *et al.,* 2003	Aggr	Muller *et al*., 1996: frozen samples: 13 Aggrs vs. 6 Superfic: Aggr = Superfic (RT-PCR, IHC) Sharma *et al.*, 2003: case report: Aggr = adjoining tissue (IHC)	Aggr = Super	Aggr < Ref, Super < Ref
TPI1	Triosephosphate isomerase	Kraljevic Pavelic *et al.*, 2009	Superic	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Aggr > Ref, Super > Ref
TRO	Trophinin	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
VSNL1	Visinin-like 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
WISP1	WNT1 inducible signaling pathway protein 1	Skubitz and Skubitz, 2004	Aggr	74: frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref
WNT5A	Wingless-type MMTV integration site family, member 5A	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super > Ref, Super < Aggr
WT1	Wilms tumor 1	Nik *et al.*, 2005	Aggr	cell culture: 5 Aggrs vs 5 Fascia: Aggr > Ref (real-time RT-PCR, northern, western, IHC)	Aggr > Ref	Aggr > Ref
ZIC1	Zic family member 1 (odd-paired homolog, Drosophila)	Denys *et al.,* 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr > Ref, Super < Aggr

Gene list D: Gene expression studies published in the literature that could not be confirmed by our own data

in Gene lists A or B

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
ACP5	acid phosphatase 5, tartrate resistant (TRAP)	Pan *et al.*, 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
ACTA2	Actin, alpha 2, smooth muscle, aorta	Bisson *et al*., 2003; Kopp *et al.,* 2006; Pagnotta *et al*., 2003; Lubahn *et al.*, 2007; Tomasek *et al.*, 1995; Berndt *et al.*, 1995; Lee *et al.,* 2006	Super	Bisson *et al.*, 2003: cell culture: nodule and cord superfic fibroblasts vs flexor retinaculum fibroblasts: Superfic > Ref (IHC) Kopp *et al*., 2006: cell culture: cord superfic fibroblasts vs. control tissue fibroblasts: Superfic > Ref (Western) Pagnotta *et al.*, 2003: cell culture: nodule superfic fibroblasts (10 patients) vs normal palmar fasica fibroblasts (4) : Superfic > Ref (IHC) Lubahn *et al.*, 2007: frozen tissues: superfic (25 samples) vs control tissue (5 samples): Superfic > Ref (IHC) Tomasek *et al*., 1995: cell culture: nodule superfic fibroblasts (11 patients) vs normal palmar fascia fibroblasts: Superfic > Ref (IHC) Berndt *et al*., 1995: frozen tissues: Active proliferative palmar noduli superfic vs. surrounding aponeurotic tissues: Superfic > Ref (ISH) Lee *et al.*, 2006 : frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array)	Super > Ref	Aggr > Ref, Super < Aggr
ACTG1	Actin, cytoplasmic 2	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
ADAM19	ADAM metallopeptidase domain 19 (meltrin beta)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif, 14	Johnston *et al*., 2007	Super	frozen tissues: nodule superfic (? samples) vs normal palmar fascia (?): Superfic > Ref (realtime RT-PCR)	Super > Ref	not differentially expressed
AKT	v-akt murine thyoma viral oncogene homolog	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (Western)	Super > Ref	not differentially expressed
ALCAM	Activated leukocyte cell adhesion molecule	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3 (ALDH6)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Super > Aggr
ALDH5A1	Aldehyde dehydrogenase 5 family, member A1 (succinate-semialdehyde dehydrogenase)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
ARCN1	Archain 1 (archain vesicle transport protein 1; coatomer delta subunit)	Pan *et al.*, 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	Qian *et al.*, 2004; Kraljevic Pavelic *et al.*, 2009	Super	Qian *et al*., 2004: frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray) Kraljevic Pavelic *et al.*, 2009: frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
ARL4C	ADP-ribosylation factor like 4C	Zhang *et al*., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
ATF5	Activating transcription factor 5	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
ATP7B	ATPase, Cu++ transporting, beta polypeptide	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
BASP1	Brain abundant membrane attached signal protein 1	Zhang *et al*., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
BGN	Biglycan (bone/cartilage proteoglycan-I; dermatan sulphate proteoglycan I)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
BMP4	Bone morphogenetic protein 4	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic < Ref (RT-PCR, Western, IHC)	Super < Ref	not differentially expressed
BMP6	Bone morphogenetic protein 6	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic < Ref (RT-PCR)	Super < Ref	not differentially expressed
BMP7	Bone morphogenetic protein 7	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic = Ref (no expression) (RT-PCR)	Super = Ref	Aggr > Ref, Super > Ref, Super < Aggr
BMPR1A	Bone morphogenetic protein receptor 1A	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic < Ref (RT-PCR)	Super < Ref	not differentially expressed
BMPR1B	Bone morphogenetic protein receptor 1B	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic < Ref (RT-PCR)	Super < Ref	not differentially expressed
BMPR2	Bone morphogenetic protein receptor 2	Shin *et al.*, 2004	Super	cell culture: nodule and cord superfic fibroblasts vs normal palmar fascia fibroblasts: Superfic < Ref (RT-PCR)	Super < Ref	not differentially expressed
BRF2	Subunit of RNA polymerase III transcription initiation factor, BRF1-like	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
C20orf103	Chromosome 20 open reading frame 103	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
CALR	Calrecticulin	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
CCL5	Chemokine (C-C motif) ligand 5 (RANTES)	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
CCT2	T-complex protein 1 subunit beta	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
CD14	CD14 antigen	Pan *et al.*, 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	Aggr > Ref
CD81	CD81 antigen (target of antiproliferative antibody 1)	Pan *et al.*, 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, WAF1, Cip1)	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (Western)	Super > Ref	not differentially expressed
CEECAM1	Cerebral endothelial cell adhesion molecule 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
CLEC3B	C-type lectin domain family 3, member B (tetranectin; plasminogen binding protein)	Pan *et al*., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	Aggr < Ref, Super > Aggr

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
CNTN1	Contactin 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
CNTN1	Contactin 1, mRNA (cDNA clone MGC:41894 IMAGE:5273941)	Lee *et al.*, 2006	Super	frozen tissues: cord superfic (4) vs. superfic-adjacent control fascia (4) vs. normal palmar fascia (3): Superfic > Ref (cDNA Array)	Super > Ref	not differentially expressed
COL15A1	Collagen, type XV, alpha 1	Satish *et al.*, 2008	Super	cell cultures superficial fibromatosis vs. normal fibroblasts (2 microarray platforms)	Super < Ref	not differentially expressed
COMP	Cartilage oligomeric matrix protein	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	Aggr < Ref, Super > Aggr
CREBL1	cAMP responsive element binding protein-like 1	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
CRYAB	Crystallin, alpha B	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
CRYAB	Crystallin, alpha B	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
CSF	Colony stimulating factor 2 (granulocyte-macrophage GM-CSF)	Qian *et al*., 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
CTNNA1	Catenin (cadherin-associated protein), alpha 1 (alpha-catenin)	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Carlson and Fletcher, 2007; Montgomery *et al*., 2001; Varallo *et al*., 2003	Super	Carlson and Fletcher, 2007: 25 cases of superficial fibromatoses: nuclear positivity present in 14 cases (IHC). Montgomery *et al.*, 2001: 22 palmar, 5 plantar cases of superficial fibromatoses: Nuclear accumulation of β-catenin present in 86% of superficial fibromatosis cases ranging from 5 100% of nuclei (IHC). Varallo *et al*., 2003: Several patient-matched superficial fibromatosis specimens: lesional tissues expressed increasing amounts of β-catenin compared to normal appearing control fascia (Western, IHC).	Super > Ref	not differentially expressed
CTTN	Cortactin	Qian *et al.,* 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1 SDF1)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Super > Ref, Super > Aggr
DAD1	Defender against cell death 1	Qian *et al.,* 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
DES	Desmin	Kosmehl *et al.*, 1995; Berndt *et al.*, 1995	Super	Kosmehl *et al.*, 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC) Berndt *et al.*, 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC)	Super > Ref	not differentially expressed
ECHS1	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	Pan *et al.*, 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
ENG	Endoglin (Osler-Rendu-Weber syndrome 1)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (Western)	Super > Ref	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
EPB49	Erythrocyte membrane protein band 4.9 (dematin)	Pan *et al*., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
FBLN1	Fibulin-1	Satish *et al.*, 2008	Super	cell cultures superficial fibromatosis vs. normal fibroblasts (2 microarray platforms)	Super < Ref	not differentially expressed
FGF2	Fibroblast growth factor 2 (bFGF)	Lappi *et al.*, 1992; Gonzalez *et al.*, 1992; Berndt *et al*., 1995	Super	Lappi *et al*., 1992: cell culture: Superfic expresses FGF2 (northern) Gonzalez *et al.*, 1992: 9 superfic vs 3 normal fascia: Superfic > Ref (ISH) Berndt *et al*., 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (ISH, IHC)	Super > Ref	not differentially expressed
FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	Gonzalez *et al.*, 1992	Super	9 superfic vs 3 normal fascia: Superfic > Ref (ISH)	Super > Ref	not differentially expressed
FLNB	Filamin B, beta (actin binding protein 278)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
GARS	Glycyl-tRNA synthetase	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
GAS1	Growth arrest-specific 1	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
GNAS	GNAS complex locus (G-s-alpha; adenylate cyclase-stimulating G alpha protein)	Qian *et al.,* 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	Aggr > Ref
GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
GSN	Gelsolin	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
HMMR	Hyaluronan-mediated motility receptor (RHAMM)	Tolg *et al.*, 2003	Aggr	frozen samples (6 Aggrs): Aggr > normal fascia (RT-PCR, IHC)	Aggr > Ref	not differentially expressed
HNRPH3	Heterogeneous nuclear ribonucleoprotein H3	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
HSPA1A	Heat shock 70kDa protein 1	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
HSPA2	Heat shock 70kDa protein 2	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
HSPA8	Heat shock cognate 71kD protein	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
HSPB1	Heat shock 27kDa protein 1	Qian *et al.,* 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
ICAM2	Intercellular adhesion molecule 2	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
IFI30	Interferon, gamma-inducible protein 30	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
IGF1R	Insulin-like growth factor 1 receptor	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (Western)	Super > Ref	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
IGF2	Insulin-like growth factor 2	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
JUNB	Jun B proto-oncogene	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
KRT10	Keratin 10	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
KRT18	Keratin 18	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
KRT7	Keratin 7	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
KRT9	Keratin 9	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
LAMB2	Laminin, beta 2 (Laminin S)	Kosmehl *et al.*, 1995	Super	nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC)	Super > Ref	Aggr < Ref (LAMC1, formerly LAMB2)
LAMB3	Laminin, beta 3	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
LEF1	Lymphoid enhancer-binding factor 1	Tejpar *et al.*, 2001	Aggr	frozen samples (10 Aggrs): Aggr = Ref (10 Fascia same patients): all are negative (RT-PCR)	Aggr = Ref	Aggr > Ref, Super > Ref, Super < Aggr
LDHB	L-lactate dehydrogenase B chain	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Super < Ref
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
MAB21L1	Mab-21-like 1	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
MALL	Mal, T-cell differentiation protein-like (BENE)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
MFAP4	Microfibrillar-associated protein 4	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Aggr > Super, Aggr > Ref
MFGE8	Milk fat globule-EGF factor 8 protein	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
MMP12	Matrix metalloproteinase 12	Denys *et al.*, 2004a	Aggr	cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR)	Aggr > Ref	not differentially expressed
MMP13	Matrix metalloproteinase 13	Denys *et al.*, 2004a	Aggr	cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR)	Aggr > Ref	not differentially expressed
MMP13	Matrix metalloproteinase 13	Johnston *et al.*, 2007	Super	frozen tissues: nodule superfic (? samples) vs normal palmar fascia (?): Superfic > Ref (realtime RT-PCR)	Super > Ref	not differentially expressed
MMP2	Matrix metalloproteinase 2	Denys *et al.*, 2004a; Kong *et al.*, 2004	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr = Ref (real time RT-PCR) Kong *et al.*, 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr = Ref (real time RT-PCR)	Aggr = Ref	Aggr > Ref, Super > Ref

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
MMP3	Matrix metalloproteinase 3	Denys *et al.*, 2004a; Kong *et al.,* 2004; Denys *et al.,* 2004b	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR) Kong *et al.*, 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr > Ref (real time RT-PCR) Denys *et al.*, 2004b: cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Aggr < Ref, Super < Ref, Super < Aggr
MMP7	Matrix metalloproteinase 7	Denys *et al.*, 2004a; Kong *et al.,* 2004; Denys *et al.,* 2004b	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr > Ref (real time RT-PCR) Kong *et al*., 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr = Ref (real time RT-PCR) Denys *et al.*, 2004b: cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
MMP7	Matrix metalloproteinase 7	Johnston *et al.,* 2007	Super	frozen tissues: nodule superfic (? samples) vs normal palmar fascia (?): Superfic (nodule and cord) > Ref (realtime RT-PCR)	Super > Ref	not differentially expressed
MXRA5	Matrix-remodelling associated 5 (adlican)	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
MYC	v-myc myelocytomatosis viral oncogene homolog (avian) (c-myc)	Jemec *et al.*, 1999	Super	paraffin blocks: superfic (21 samples) vs recurrent superfic (9) vs normal fascia (6) vs flexor retinaculum (12): superfic > recurrent superfic = normal fascia > flexor retinaculum (negative!) (IHC)	Super > Ref	Aggr < Ref, Super < Ref
MYL3	Myosin, light polypeptide 3	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
MYL6	Myosin, light polypeptide 6	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
MYL9	Myosin, light polypeptide 9, regulatory (MYRL2)	Denys *et al.*, 2004b	Aggr	76: cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
NADP	Isocitrate dehydrogenase (NADP)	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
NCAM1	Neural cell adhesion molecule	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
NID2	Nidogen 2 (osteonidogen)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Aggr > Ref, Super > Ref, Super > Aggr
PARK7	Onocgene DJI	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PCBP1	Poly(rC)-binding protein 1	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PDGFB	Platelet-derived growth factor beta	Alman *et al.,* 1995	Aggr	frozen samples: Aggr, fibrous displasia, recurrent Superfic > Superfic, plantar fascia, mature scar (RT-PCR, ISH)	Aggr > Super	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
PDGFB	Platelet-derived growth factor beta	Terek *et al.*, 1995; Alman *et al.*, 1996; Tarpila *et al.*, 1996; Hindman *et al.,* 2003	Super	Terek *et al*., 1995: Superfic (6 cases) vs normal fascia (? cases): Superfic > Ref (RT-PCR, southern) Alman *et al.*, 1996: cell cultures: palmar superfic (8 cases) vs uninvolved palmar fascia (4) vs normal palmar fascia (4): cyclic mechanical strain to these cultures: PDGFA expression increased in Superfic and uninvolved palmar tissue. PDGFB was increased only in Superfic. Normal fascia did not express any PDGF. Tarpila *et al.*, 1996 : cell cultures: nodular superfic (10 cases, late stage) vs forearm control fibroblasts (? cases): contraction of collagen latices: control fibroblasts > superfic ! PDGFB, but not PDGFA, increased chemotactic activity in superfic and control fibroblasts Hindman *et al.*: cell culture (5 superfic): PDGFB treatement: expression of ACTA2 decreases (western), but proliferation increases	Super > Ref	not differentially expressed
PGK1	Phosphoglycerate kinase 1	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PKM2	Pyruvate kinase isoenzymes M1/M2	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PENK	Proenkephalin	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
PLS3	Plastin 3	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PODXL	Podocalyxin-like	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
PRC1	Protein regulator of cytokinesis	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
PRDXI	Peroxiredoxin	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
PRG1	Proteoglycan 1, secretory granule	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Super < Ref
PRKX	Protein kinase, X-linked	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
PTK7	Protein tyrosine kinase 7	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
QSCN6	Quiescin Q6	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
RACK1	Lung cancer oncogene 7	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
RB1	Retinoblastoma 1 (including osteosarcoma)	Muller *et al.*, 1996; Hoos *et al.*, 2001	Aggr	Muller *et al*., 1996: frozen samples: 13 Aggrs vs. 6 Superfic: Aggr < Superfic (RT-PCR, IHC) Hoos *et al*., 2001: frozen samples, paraffin blocks: 24 Aggrs vs 39 Fibrosarcoma (LG, HG): Fibrosarcoma < Aggr (IHC)	Aggr < Super	Aggr > Ref
RB1	Retinoblastoma 1 (including osteosarcoma)	Muller *et al.*, 1996	Super	Palmar superfic (6 cases) vs Aggr (13 cases): Superfic > Aggr (IHC, RT-PCR)	Super > Aggr	Aggr > Ref
RCAN	Regulator of calcineurin	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
RGS3	Regulator of G protein signalling 3	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
RHOA	Ras homolog gene family, member A	Qian *et al.,* 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Super > Ref
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1 = PAI1), member 1	Li *et al.*, 2005	Aggr	frozen samples: 6 Aggrs vs 6 normal fascia: Aggr > Ref (RT-PCR, IHC)	Aggr > Ref	not differentially expressed
SERPINF1	Pigment epithelium-derived factor	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
SFRP1	Secreted frizzled-related protein 1	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
SGCD	Sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
SHOX2	Short stature homeobox 2	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
SLC7A8	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	not differentially expressed
SORCS1	Sortilin-related VPS10 domain containing receptor 1	Skubitz and Skubitz, 2004	Aggr	frozen samples (12 Aggrs): Aggr > 16 non-neoplastic tissues (Affymetrix array)	Aggr > Ref	not differentially expressed
SOX4	SRY (sex determining region Y)-box 17	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
SPARCL1	SPARC-like 1 (mast9, hevin)	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr > Ref (Affymetrix array)	Aggr > Ref	Super > Aggr
STIP1	Stess-induced-phosphoprotein 1	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
SYMPK	Symplekin	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed
TAGLN	Transgelin	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	Super > Aggr
TCF4	Transcription factor 4 (SEF2-1B; not (!!) TCF7L2)	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic > Ref (Atlas microarray)	Super > Ref	Aggr > Ref
TFPI2	Tissue factor pathway inhibitor 2	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	Aggr > Ref, Super < Aggr

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
TGFB1	Transforming growth factor beta 1	Badalamente *et al.*, 1996; Bisson *et al.*, 2003; Kopp *et al.*, 2006; Berndt *et al.*, 1995; Hindman *et al.*, 2003; Zhang *et al.*, 2008	Super	Badalamente *et al.*, 1996: Superfic (? cases): high intracellular expression in fibroblasts, myofibroblasts and endothelial cells in all superfic samples, regardless of disease stage. It is not present in the ECM (IHC). Cell cultures with superfic myofibroblasts: addition of TGFB1: proliferation increases, but TGFB1 < TGFB2. Bisson *et al.*, 2003: cell culture: nodular superfic vs cord superfic: TGFB1 induces expression of ACTA2 in both cultures to a similar level, although the starting point was different: cord < nodul. Kopp *et al.*, 2006: cell culture: superfic (? cases) vs. normal tendon pully fibroblasts: Superfic > Ref (Luciferase-assay) 34A: cell culture: Superfic vs. flexor retinaculum fibroblasts: Addition of TGFB1 (low dose): increase in ACTA2+ myofibroblasts in Superfic culture. Addition of high dose: contrary Berndt *et al.*, 1995: nodular palmar superfic (? cases): proliferative noduli > surrounding aponeurosis (IHC) Hindman *et al.*, 2003: cell culture (5 superfic): TGFB1 treatement: expression of ACTA2 increases (western), but proliferation does not change Zhang *et al.*, 2008: cord superfic vs. superfic-adjacent control fasci	Super > Ref	not differentially expressed
TGFBR1	Transforming growth factor beta receptor type 1	Locci *et al.*, 2001	Aggr	cell culture: Aggr fibroblasts vs. normal fibroblasts: Aggr > Ref (Western)	Aggr > Ref	not differentially expressed
TGFBR2	Transforming growth factor beta receptor type 2	Locci *et al.*, 2001	Aggr	cell culture: Aggr fibroblasts vs. normal fibroblasts: Aggr > Ref (Western)	Aggr > Ref	Aggr < Ref
TGFBR3	Transforming growth factor beta receptor type 3	Locci *et al.*, 2001	Aggr	cell culture: Aggr fibroblasts vs. normal fibroblasts: Aggr = Ref (Western)	Aggr = Ref	Aggr < Ref, Super < Ref
TIMP1	Metallopeptidase inhibitor 1 (tissue inhibitor of metalloproteinase 1)	Denys *et al.*, 2004a; Kong *et al.*, 2004	Aggr	Denys *et al.*, 2004a: cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr < Ref (real time RT-PCR) Kong *et al.*, 2004: cell culture: 9 Aggrs from APC-KO-mice vs. 9 normal fascia from WT-mice: Aggr < Ref (real time RT-PCR)	Aggr < Ref	not differentially expressed
TIMP1	Metallopeptidase inhibitor 1 (tissue inhibitor of metalloproteinase 1)	Ulrich *et al.*, 2003; Johnston *et al.*, 2007	Super	Ulrich *et al.*, 2003: Sera and tissue samples of 22 superfic vs sera and palmar fascia of 20 healthy controls: Superfic > Ref (ELISA for sera, IHC for tissue samples) Johnston *et al.*, 2007: frozen tissues: nodule superfic (? samples) vs normal palmar fascia (?): Superfic > Ref (realtime RT-PCR)	Super > Ref	not differentially expressed
TIMP2	Metallopeptidase inhibitor 2 (tissue inhibitor of metalloproteinase 2)	Denys *et al.*, 2004a	Aggr	cell culture: 7 Aggrs vs. 7 matched Fascia: Aggr = Ref (real time RT-PCR)	Aggr = Ref	Aggr > Ref, Super > Ref
TP53	Tumor protein p53 (Li-Fraumeni syndrome)	Kraljevic Pavelic *et al.*, 2009	Super	tissue Super vs. normal fascia: Super > Ref (Western)	Super > Ref	Aggr < Ref, Super < Ref
TMEM49	Transmembrane protein 49	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
TMEM158	Transmembrane protein 158	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed
TMSB10	Thymosin, beta 10 (migration-inducing gene 12)	Qian *et al.*, 2004	Super	frozent tissues: 9 superfic palmar fasica vs adjacent normal tendon: Superfic > Ref (Atlas microarray)	Super > Ref	Aggr > Ref
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	Super > Ref, Super > Aggr
TNFSF12	Tumor necrosis factor (ligand) superfamily, member 12	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic < Ref (Atlas microarray)	Super < Ref	not differentially expressed

Gene		Paper	Literature	Paper		Own results
				Experiment description	Statement	
TRH	Thyrotropin-releasing hormone	Denys *et al.*, 2004b	Aggr	cell culture: 4 Aggrs vs. 4 Fasica: Aggr < Ref (Affymetrix array)	Aggr < Ref	not differentially expressed
TUBA3	Tubulin, alpha 3	Pan et al., 2003	Super	frozen tissues: 6 superfic palmar fascia vs 2 Ref (normal palmar fascia): Superfic > Ref (Atlas microarray)	Super > Ref	not differentially expressed
VCP	Transitional endoplasmic reticulum ATPase (valosin-containing protein)	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
VIM	Vimentin	Kraljevic Pavelic *et al.*, 2009	Super	frozen tissues: 12 cases disease vs. control (2D-gelelectrophoresis, MS)	Super > Ref	not differentially expressed
XRCC1	X-ray repair complementing defective repair	Zhang et al., 2008	Super	Dupuytren's diseased cord tissue vs. normal transverse ligament of palmar fascia: Super > Ref (cDNA microarray)	Super > Ref	not differentially expressed

Gene list E: WNT5A-induced differentially expressed genes in Aggr6

Ratio = WNT5A-treated cells vs. untreated (control) cells
WNT5A = absolute expression of the gene after WNT5A treatment
control = absolute expression of the gene in untreated cells

1) Proliferation

Gene		Ratio	P-value	WNT5A	control
ANKRD15	ankyrin repeat domain 15	2.72	2.92E-09	4.52	1.70
APPBP1	amyloid beta precursor protein binding protein 1	3.57	4.21E-12	1.66	0.48
APPL1	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper conta	2.66	5.24E-09	0.76	0.29
APPL2	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper conta	3.87	7.17E-13	0.65	0.17
AREG	amphiregulin (schwannoma-derived growth factor)	0.02	1.24E-22	0.00	0.18
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	14.20	1.82E-20	2.11	0.15
ATR	ataxia telangiectasia and Rad3 related	2.61	8.41E-09	0.56	0.22
AURKA	aurora kinase A	9.18	8.15E-19	1.43	0.16
AURKB	aurora kinase B	5.99	2.21E-16	0.39	0.07
AXIN1	axin 1	0.48	2.64E-06	0.07	0.15
BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	0.37	4.44E-09	2.48	6.81
BBC3	BCL2 binding component 3	4.18	1.41E-13	1.65	0.41
BCL2L11	BCL2-like 11 (apoptosis facilitator)	0.10	2.72E-19	0.03	0.28
BCL2L12	BCL2-like 12 (proline rich)	2.23	4.98E-07	1.07	0.49
BCL7A	B-cell CLL/lymphoma 7A	2.14	1.34E-06	0.39	0.19
BCOR	BCL6 co-repressor	9.37	8.01E-19	0.20	0.02
BDKRB2	bradykinin receptor B2	6.25	1.28E-16	0.20	0.03
BDNF	brain-derived neurotrophic factor	0.36	1.26E-09	0.11	0.32
BDNF	brain-derived neurotrophic factor	0.40	2.96E-08	0.64	1.64
BIRC2	baculoviral IAP repeat-containing 2	2.51	2.22E-08	2.37	0.97
BIRC5	baculoviral IAP repeat-containing 5 (survivin)	3.98	0	6.56	1.70
BMP6	bone morphogenetic protein 6	0.07	2.52E-20	0.01	0.12
BMPER	BMP binding endothelial regulator	2.28	3.02E-07	0.16	0.07
BRCA1	breast cancer 1, early onset	24.55	0	0.21	0.01
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	9.57	5.33E-19	1.02	0.11
BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	10.18	2.97E-19	0.48	0.05
BUB3	BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)	7.68	6.37E-18	7.02	0.94
CAPRIN1	cell cycle associated protein 1	3.15	7.63E-11	2.63	0.86
CAPRIN2	caprin family member 2	2.15	1.20E-06	0.32	0.15
CCL2	chemokine (C-C motif) ligand 2	2.03	1.40E-45	0.16	0.08
CCNA2	cyclin A2	14.35	0	1.13	0.08
CCNB1	cyclin B1	6.63	4.61E-17	6.28	0.97
CCNB2	cyclin B2	5.14	2.62E-15	6.88	1.37
CCND1	cyclin D1	2.04	0	7.12	3.58
CCNE1	cyclin E1	0.40	3.19E-08	0.46	1.16
CCNG2	cyclin G2	5.31	1.52E-15	0.89	0.17
CCNL1	cyclin L1	0.30	2.64E-11	0.82	2.78
CCPG1	cell cycle progression 1	0.49	0	0.22	0.46
CCRK	cell cycle related kinase	0.45	5.97E-07	0.08	0.18
CDC16	cell division cycle 16 homolog (S. cerevisiae)	2.77	1.83E-09	2.42	0.90
CDC2	cell division cycle 2, G1 to S and G2 to M	5.27	0	1.77	0.34
CDC20	cell division cycle 20 homolog (S. cerevisiae)	7.22	1.45E-17	0.56	0.08
CDC23	cell division cycle 23 homolog (S. cerevisiae)	3.51	6.17E-12	0.58	0.17
CDC25A	cell division cycle 25 homolog A (S. pombe)	0.31	3.13E-11	0.10	0.32
CDC25B	cell division cycle 25 homolog B (S. pombe)	3.15	8.04E-11	1.50	0.49
CDC25C	cell division cycle 25 homolog C (S. pombe)	22.64	1.96E-21	0.16	0.01
CDC37L1	cell division cycle 37 homolog (S. cerevisiae)-like 1	2.88	7.12E-10	0.34	0.12
CDC40	cell division cycle 40 homolog (S. cerevisiae)	2.65	6.23E-09	0.24	0.09

Gene		Ratio	P-value	WNT5A	control
CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	3.59	3.71E-12	2.09	0.60
CDC7	cell division cycle 7 homolog (S. cerevisiae)	28.81	8.36E-22	0.16	0.01
CDCA2	cell division cycle associated 2	3.83	8.92E-13	2.16	0.58
CDCA3	cell division cycle associated 3	5.07	3.48E-15	0.49	0.10
CDCA5	cell division cycle associated 5	3.76	1.33E-12	2.34	0.64
CDCA7L	cell division cycle associated 7-like	3.59	3.81E-12	0.62	0.18
CDCA8	cell division cycle associated 8	8.99	1.02E-18	4.26	0.49
CDK5RAP1	CDK5 regulatory subunit associated protein 1	2.21	6.14E-07	0.23	0.11
CDK5RAP2	CDK5 regulatory subunit associated protein 2	2.20	6.60E-07	0.84	0.39
CDK6	cyclin-dependent kinase 6	2.45	4.42E-08	3.60	1.51
CDK8	cyclin-dependent kinase 8	2.30	2.25E-07	0.43	0.19
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	4.50	0	0.15	0.03
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	0.45	0	0.09	0.21
CDKN2AIP	CDKN2A interacting protein	0.44	3.25E-07	0.25	0.57
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	5.86	2.93E-16	2.82	0.49
CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	2.66	5.32E-09	3.85	1.49
CGRRF1	cell growth regulator with ring finger domain 1	0.40	2.54E-08	0.21	0.53
CHAF1A	chromatin assembly factor 1, subunit A (p150)	4.51	3.00E-14	1.44	0.33
CIZ1	CDKN1A interacting zinc finger protein 1	0.48	3.50E-06	0.44	0.93
CKS1B	CDC28 protein kinase regulatory subunit 1B	4.06	2.50E-13	7.75	1.96
CUL1	cullin 1	4.07	2.43E-13	0.82	0.21
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.40	3.26E-08	0.09	0.24
CXCL2	chemokine (C-X-C motif) ligand 2	0.06	1.03E-20	0.02	0.30
CXCL3	chemokine (C-X-C motif) ligand 3	0.03	2.86E-22	0.04	1.32
CXCR7	Homo sapiens chemokine (C-X-C motif) receptor 7 (CXCR7), transcript variant 1,	0.21	1.03E-14	0.08	0.41
DCLRE1A	DNA cross-link repair 1A (PSO2 homolog, S. cerevisiae)	8.39	4.05E-18	0.10	0.01
DKK1	dickkopf homolog 1 (Xenopus laevis)	0.17	1.74E-16	6.17	38.43
DLG7	discs, large homolog 7 (Drosophila)	5.86	2.95E-16	1.43	0.25
DMTF1	cyclin D binding myb-like transcription factor 1	2.57	1.27E-08	0.86	0.35
DTYMK	deoxythymidylate kinase (thymidylate kinase)	2.25	3.71E-07	21.30	9.71
E2F6	E2F transcription factor 6	0.36	1.78E-09	0.30	0.87
E2F7	E2F transcription factor 7	3.24	3.85E-11	3.02	0.96
EAPP	E2F-associated phosphoprotein	3.61	3.15E-12	1.43	0.41
EDNRA	endothelin receptor type A	3.66	3.40E-12	0.11	0.03
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene	2.24	0	0.34	0.16
EGR1	early growth response 1	5.97	2.22E-16	1.03	0.18
ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	11.70	9.80E-20	0.21	0.02
FANCD2	Fanconi anemia, complementation group D2	2.67	4.90E-09	0.50	0.19
FGF18	fibroblast growth factor 18	0.05	2.00E-21	0.01	0.23
FGF2	fibroblast growth factor 2 (basic)	3.13	0	2.31	0.76
FGF5	fibroblast growth factor 5	6.57	6.23E-17	0.21	0.03
FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	0.15	5.35E-17	0.09	0.59
FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	0.26	7.53E-13	0.28	1.12
FGFBP3	fibroblast growth factor binding protein 3	0.32	1.06E-10	0.04	0.12
FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndron	0.07	1.16E-20	0.02	0.35
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0.01	4.77E-23	0.05	4.74
FST	follistatin	0.37	2.66E-09	0.85	2.39
FZD2	frizzled homolog 2 (Drosophila)	3.38	1.47E-11	1.70	0.52
FZD4	frizzled homolog 4 (Drosophila)	4.19	1.39E-13	0.28	0.07
FZD5	frizzled homolog 5 (Drosophila)	0.29	1.03E-11	0.04	0.14
FZD6	frizzled homolog 6 (Drosophila)	2.14	1.47E-06	1.21	0.58
FZD7	frizzled homolog 7 (Drosophila)	8.84	1.23E-18	1.67	0.19
FZD8	frizzled homolog 8 (Drosophila)	0.47	2.23E-06	0.06	0.14
GADD45B	growth arrest and DNA-damage-inducible, beta	12.07	6.22E-20	6.51	0.55
GAS1	growth arrest-specific 1	3.93	5.17E-13	0.31	0.08
GPS2	G protein pathway suppressor 2	2.09	3.08E-06	0.12	0.06

Gene		Ratio	P-value	WNT5A	control
H2AFX	H2A histone family, member X	4.53	2.79E-14	23.57	5.35
HBEGF	heparin-binding EGF-like growth factor	0.07	2.17E-20	0.02	0.32
HBEGF	heparin-binding EGF-like growth factor	0.06	1.19E-20	0.01	0.15
HDAC4	histone deacetylase 4	4.73	2.04E-14	0.10	0.02
HRASLS3	HRAS-like suppressor 3	0.40	2.95E-08	1.44	3.68
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	2.07	3.24E-06	0.37	0.18
IFNE1	interferon epsilon 1	0.38	5.97E-09	0.06	0.15
IGF1R	insulin-like growth factor 1 receptor	0.34	0	0.04	0.12
IGFBP3	insulin-like growth factor binding protein 3	0.32	7.54E-11	1.12	3.64
IL11	interleukin 11	0.06	8.03E-21	1.52	25.11
IL13RA2	interleukin 13 receptor, alpha 2	2.40	7.69E-08	0.99	0.43
IL1RAP	interleukin 1 receptor accessory protein	0.13	7.61E-18	0.07	0.57
IL20RB	interleukin 20 receptor beta	0.29	1.23E-11	0.06	0.23
IL6	interleukin 6 (interferon, beta 2)	0.44	0	0.56	1.29
IL8	interleukin 8	0.04	1.14E-21	0.01	0.19
INCENP	inner centromere protein antigens 135/155kDa	8.39	2.22E-18	1.57	0.19
ING4	inhibitor of growth family, member 4	4.61	2.64E-14	0.13	0.03
JAG1	jagged 1 (Alagille syndrome)	0.18	5.86E-16	0.03	0.17
KIAA1787	KIAA1787 protein	3.07	1.78E-10	0.14	0.05
KIF11	kinesin family member 11	24.79	9.22E-22	0.54	0.02
KIF23	kinesin family member 23	8.14	3.16E-18	4.38	0.55
KNTC1	kinetochore associated 1	4.95	0	0.47	0.10
LZTS1	leucine zipper, putative tumor suppressor 1	4.95	5.34E-15	0.53	0.11
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	3.14	8.71E-11	1.80	0.59
MAPK7	mitogen-activated protein kinase 7	2.80	1.44E-09	0.39	0.14
MCM7	minichromosome maintenance complex component 7	2.17	1.04E-06	3.76	1.78
MCM8	minichromosome maintenance complex component 8	4.36	6.16E-14	0.38	0.09
MDC1	mediator of DNA damage checkpoint 1	4.48	3.64E-14	0.37	0.08
MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	0.36	0	0.04	0.12
MDM4	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)	0.39	1.16E-08	0.08	0.22
MINA	MYC induced nuclear antigen	3.94	5.14E-13	0.26	0.07
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	2.13	1.58E-06	4.74	2.29
MPHOSPH1	M-phase phosphoprotein 1	40.56	2.30E-22	0.22	0.01
MPHOSPH8	M-phase phosphoprotein 8	3.39	1.35E-11	0.64	0.19
MPHOSPH9	M-phase phosphoprotein 9	5.76	4.75E-16	0.19	0.03
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	3.06	0	1.55	0.52
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	2.61	0	5.63	2.22
NCAPD2	non-SMC condensin I complex, subunit D2	5.27	1.72E-15	0.72	0.14
NCAPG	non-SMC condensin I complex, subunit G	16.08	7.99E-21	10.40	0.66
NEK3	NIMA (never in mitosis gene a)-related kinase 3	2.55	1.61E-08	0.37	0.15
NOLC1	nucleolar and coiled-body phosphoprotein 1	0.37	3.49E-09	1.80	4.99
PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	4.55	2.58E-14	2.26	0.51
PARD3	par-3 partitioning defective 3 homolog (C. elegans)	0.37	4.67E-09	0.04	0.12
PDGFA	platelet-derived growth factor alpha polypeptide	2.07	3.39E-06	1.85	0.92
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	6.41	7.60E-17	0.82	0.13
PDRG1	p53 and DNA damage regulated 1	0.33	2.04E-10	0.24	0.74
PKMYT1	protein kinase, membrane associated tyrosine/threonine 1	3.59	3.71E-12	1.60	0.46
PMS1	PMS1 postmeiotic segregation increased 1 (S. cerevisiae)	6.21	1.24E-16	0.49	0.08
PPARD	peroxisome proliferator-activated receptor delta	2.29	2.40E-07	1.30	0.58
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	3.47	1.09E-11	0.11	0.03
PTGFR	prostaglandin F receptor (FP)	6.23	1.70E-16	0.12	0.02
PTP4A1	protein tyrosine phosphatase type IVA, member 1	0.22	2.97E-14	0.22	1.03
PTTG1	pituitary tumor-transforming 1	2.16	1.08E-06	13.88	6.60
PYCARD	PYD and CARD domain containing	0.45	3.94E-07	0.28	0.64
RAD17	RAD17 homolog (S. pombe)	4.34	6.55E-14	1.97	0.47
RAD21	RAD21 homolog (S. pombe)	2.19	8.06E-07	4.28	2.01

Gene		Ratio	P-value	WNT5A	control
RASSF1	Ras association (RalGDS/AF-6) domain family 1	2.81	1.34E-09	2.62	0.96
RBBP6	retinoblastoma binding protein 6	2.40	7.59E-08	0.91	0.39
RBBP7	retinoblastoma binding protein 7	2.49	2.71E-08	13.50	5.56
RBL2	retinoblastoma-like 2 (p130)	2.38	1.14E-07	0.11	0.05
RCC2	regulator of chromosome condensation 2	3.05	1.74E-10	1.97	0.67
RGS2	regulator of G-protein signaling 2, 24kDa	0.39	1.44E-08	0.29	0.75
SASH1	SAM and SH3 domain containing 1	4.93	5.92E-15	0.36	0.07
SCAPER	S phase cyclin A-associated protein in the ER	3.80	1.30E-12	0.17	0.05
SMC1A	structural maintenance of chromosomes 1A	2.68	4.55E-09	0.51	0.20
SMC4	structural maintenance of chromosomes 4	4.13	1.76E-13	4.47	1.11
SNF1LK	SNF1-like kinase	0.50	6.63E-06	0.49	1.00
SPAG5	sperm associated antigen 5	4.97	4.79E-15	3.93	0.81
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	0.36	1.89E-09	0.17	0.48
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	3.19	5.93E-11	16.97	5.47
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	0.39	2.31E-08	0.04	0.11
TP53AP1	TP53 activated protein 1	0.40	3.05E-08	0.37	0.93
TP53BP2	tumor protein p53 binding protein, 2	0.25	4.95E-13	0.59	2.38
TP53I13	tumor protein p53 inducible protein 13	0.49	5.11E-06	0.05	0.11
TRIM13	tripartite motif-containing 13	3.29	3.38E-11	0.15	0.05
U2AF1	U2 small nuclear RNA auxiliary factor 1	0.13	5.94E-18	0.06	0.50
UBE2C	ubiquitin-conjugating enzyme E2C	3.59	3.64E-12	13.46	3.85
UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1	2.68	6.09E-09	0.10	0.04
VASH1	vasohibin 1	2.38	1.04E-07	0.14	0.06
VEGFA	vascular endothelial growth factor A	0.21	1.59E-14	0.06	0.29
VEGFA	vascular endothelial growth factor A	0.41	0	1.36	3.36
VEGFA	vascular endothelial growth factor A	0.30	2.10E-11	0.53	1.82
WNT5B	wingless-type MMTV integration site family, member 5B	3.16	7.08E-11	1.46	0.47
ZWINT	Homo sapiens ZW10 interactor (ZWINT), transcript variant 4, mRNA [NM_00100	4.40	4.80E-14	2.85	0.67

2) Intracellular signalling pathway components

Gene		Ratio	P-value	WNT5A	control
ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related g	0.02	7.45E-23	0.23	15.19
AKAP12	A kinase (PRKA) anchor protein (gravin) 12	0.48	2.63E-06	0.31	0.66
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	0.46	8.38E-07	0.08	0.17
ARHGAP11A	Rho GTPase activating protein 11A	9.41	9.85E-19	0.13	0.01
ARHGAP18	Rho GTPase activating protein 18	6.18	1.38E-16	0.36	0.06
ARHGAP20	Rho GTPase activating protein 20	0.30	2.68E-11	0.03	0.10
ARHGAP22	Rho GTPase activating protein 22	3.90	5.90E-13	0.99	0.26
ARHGAP24	Rho GTPase activating protein 24	7.42	1.43E-17	0.14	0.02
ARHGAP29	Rho GTPase activating protein 29	2.75	2.55E-09	0.15	0.06
ARHGEF10L	Rho guanine nucleotide exchange factor (GEF) 10-like	2.53	2.07E-08	0.21	0.09
ARHGEF17	Rho guanine nucleotide exchange factor (GEF) 17	6.35	8.67E-17	2.58	0.42
ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	3.34	2.02E-11	0.46	0.14
ATF3	activating transcription factor 3	0.15	4.21E-17	0.10	0.69
BARD1	BRCA1 associated RING domain 1	2.25	3.85E-07	0.41	0.19
BCCIP	BRCA2 and CDKN1A interacting protein	0.49	4.81E-06	0.07	0.15
BRAF	v-raf murine sarcoma viral oncogene homolog B1	0.37	0	0.05	0.15
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	2.95	3.99E-10	1.18	0.41
CDC42EP4	CDC42 effector protein (Rho GTPase binding) 4	5.90	2.84E-16	0.35	0.06
CDC42SE1	CDC42 small effector 1	2.54	2.02E-08	0.13	0.05
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	0.43	1.22E-07	0.12	0.28
CREM	cAMP responsive element modulator	0.13	4.34E-18	0.15	1.24
CREM	cAMP responsive element modulator	0.13	5.31E-18	0.33	2.61
CREM	cAMP responsive element modulator	0.44	3.40E-07	0.12	0.28
CREM	cAMP responsive element modulator	0.11	6.97E-19	0.08	0.74

Gene		Ratio	P-value	WNT5A	control
CSK	c-src tyrosine kinase	2.55	1.70E-08	0.17	0.07
CSNK1D	casein kinase 1, delta	0.33	2.27E-10	2.45	7.58
CSNK1G1	casein kinase 1, gamma 1	0.29	9.30E-12	0.03	0.12
CSNK2A1	casein kinase 2, alpha 1 polypeptide	0.41	0	0.05	0.12
DUSP4	dual specificity phosphatase 4	0.03	3.96E-22	0.02	0.54
DVL2	dishevelled, dsh homolog 2 (Drosophila)	3.86	7.57E-13	0.75	0.20
ELK1	ELK1, member of ETS oncogene family	0.46	1.05E-06	0.08	0.19
FER	Proto-oncogene tyrosine-protein kinase FER (EC 2.7.10.2) (p94-FER) (c- FER). [	2.55	1.47E-08	0.73	0.30
GEM	GTP binding protein overexpressed in skeletal muscle	0.13	9.02E-18	0.38	2.93
GSK3A	glycogen synthase kinase 3 alpha	2.35	1.29E-07	5.17	2.26
IKIP	IKK interacting protein	2.48	3.23E-08	1.29	0.54
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	3.48	7.81E-12	0.49	0.15
MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	0.36	1.54E-09	0.51	1.46
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)	0.45	4.45E-07	0.32	0.73
MAP2K2	mitogen-activated protein kinase kinase 2	0.34	2.91E-10	0.15	0.46
MAP2K3	mitogen-activated protein kinase kinase 3	0.30	2.55E-11	0.42	1.42
MAP3K5	mitogen-activated protein kinase kinase kinase 5	2.59	1.11E-08	0.17	0.07
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	0.35	7.58E-10	0.19	0.57
MAPK11	mitogen-activated protein kinase 11	0.46	9.47E-07	0.11	0.25
MAPK14	mitogen-activated protein kinase 14	4.76	0	1.65	0.36
MAPK7	mitogen-activated protein kinase 7	2.80	1.44E-09	0.39	0.14
MAPKAP1	mitogen-activated protein kinase associated protein 1	2.10	2.36E-06	0.24	0.12
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2	0.38	5.75E-09	0.16	0.43
NFAT5	Homo sapiens nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5),	0.27	1.47E-12	0.09	0.34
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	0.18	5.77E-16	0.05	0.30
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilo	2.38	9.47E-08	0.21	0.09
NR4A2	nuclear receptor subfamily 4, group A, member 2	0.01	5.47E-23	0.00	0.21
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	0.35	6.63E-10	0.67	1.98
RAB1A	RAB1A, member RAS oncogene family	2.52	2.04E-08	17.29	7.04
RAB24	RAB24, member RAS oncogene family	0.35	7.71E-10	0.19	0.57
RAB2B	RAB2B, member RAS oncogene family	3.09	1.69E-10	0.12	0.04
RAB31	RAB31, member RAS oncogene family	2.28	2.80E-07	1.32	0.60
RAB33A	RAB33A, member RAS oncogene family	0.28	2.75E-12	0.23	0.86
RAB4A	RAB4A, member RAS oncogene family	0.47	1.72E-06	0.63	1.37
RACGAP1	Rac GTPase activating protein 1	16.19	7.64E-21	3.96	0.25
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	0.28	4.65E-12	1.69	6.16
RAN	RAN, member RAS oncogene family	2.01	7.36E-06	0.51	0.26
RANBP6	RAN binding protein 6	2.65	6.36E-09	0.15	0.06
RANGAP1	Ran GTPase activating protein 1	2.51	2.45E-08	0.18	0.07
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	2.31	2.01E-07	1.09	0.49
RASA1	RAS p21 protein activator (GTPase activating protein) 1	4.35	6.14E-14	1.64	0.39
RASSF1	Ras association (RalGDS/AF-6) domain family 1	2.81	1.34E-09	2.62	0.96
RASSF3	Homo sapiens cDNA FLJ26410 fis, clone HRT09622. [AK129920]	3.76	1.61E-12	0.22	0.06
RASSF4	Ras association (RalGDS/AF-6) domain family 4	2.70	4.53E-09	0.12	0.05
RASSF8	Ras association (RalGDS/AF-6) domain family 8	0.23	7.88E-14	0.05	0.21
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	0.20	8.63E-15	0.03	0.14
RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa ligh	2.89	0	0.45	0.16
RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa ligh	0.44	3.28E-07	1.51	3.52
RIN2	Ras and Rab interactor 2	8.57	1.74E-18	1.96	0.24
RND3	Rho family GTPase 3	0.29	0	7.75	27.75
RSU1	Ras suppressor protein 1	2.24	5.16E-07	0.14	0.06
SMAD1	SMAD family member 1	6.34	9.04E-17	0.49	0.08
SMAD3	SMAD family member 3	13.33	0	0.49	0.04
SMAD4	SMAD family member 4	2.06	0	0.24	0.12
TTRAP	TRAF and TNF receptor associated protein	3.27	3.16E-11	2.45	0.77

3) ECM / Cell adhesion

Gene		Ratio	P-value	WNT5A	control
ADAM17	ADAM 17 precursor (EC 3.4.24.86) (A disintegrin and metalloproteinase domain	0.48	2.18E-06	0.30	0.64
ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	0.27	1.29E-12	0.59	2.27
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.09	9.44E-20	1.96	23.14
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.05	2.75E-21	1.16	23.09
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif, 14	7.39	1.06E-17	0.70	0.10
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	4.38	5.34E-14	0.76	0.18
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	0.25	2.32E-13	0.31	1.30
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	2.12	1.78E-06	0.30	0.15
AJAP1	adherens junction associated protein 1	0.10	5.18E-19	0.03	0.25
ALCAM	activated leukocyte cell adhesion molecule	0.44	2.88E-07	0.05	0.11
ARF6	ADP-ribosylation factor 6	0.46	9.66E-07	1.02	2.27
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	4.63	1.86E-14	0.86	0.19
CCL2	chemokine (C-C motif) ligand 2	2.03	1.40E-45	0.16	0.08
CDH4	cadherin 4, type 1, R-cadherin (retinal)	0.47	1.31E-06	0.08	0.19
CLDN11	claudin 11 (oligodendrocyte transmembrane protein)	0.37	3.02E-09	0.24	0.66
CNTNAP1	contactin associated protein 1	0.49	4.35E-06	0.06	0.12
COL4A1	collagen, type IV, alpha 1	0.44	3.32E-07	0.05	0.11
COL5A2	collagen, type V, alpha 2	0.42	1.01E-07	0.04	0.10
COL8A2	collagen, type VIII, alpha 2	0.42	8.88E-08	0.12	0.28
CTGF	connective tissue growth factor	4.55	2.55E-14	9.65	2.18
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	0.34	4.89E-10	0.12	0.37
CTSL2	cathepsin L2	0.48	2.39E-06	0.09	0.19
DCBLD1	discoidin, CUB and LCCL domain containing 1	4.41	4.72E-14	0.62	0.14
DCBLD2	discoidin, CUB and LCCL domain containing 2	0.42	7.44E-08	0.34	0.82
DST	dystonin	0.04	5.66E-22	0.01	0.34
EDG1	endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	0.10	3.37E-19	0.11	1.11
EDIL3	EGF-like repeats and discoidin I-like domains 3	0.46	7.09E-07	0.32	0.72
EFS	embryonal Fyn-associated substrate	12.45	4.86E-20	1.73	0.14
FAT	FAT tumor suppressor homolog 1 (Drosophila)	0.49	4.37E-06	5.44	11.44
FBLIM1	filamin binding LIM protein 1	0.36	1.41E-09	0.07	0.22
FLRT2	fibronectin leucine rich transmembrane protein 2	2.11	2.33E-06	0.12	0.06
FLRT3	fibronectin leucine rich transmembrane protein 3	0.03	6.22E-22	0.00	0.12
GNE	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	4.87	7.29E-15	0.59	0.12
GP9	glycoprotein IX (platelet)	0.38	1.01E-08	0.04	0.12
GPR56	G protein-coupled receptor 56	2.83	1.09E-09	0.75	0.27
HAPLN1	hyaluronan and proteoglycan link protein 1	0.22	2.24E-14	0.24	1.12
HAS1	hyaluronan synthase 1	0.02	9.08E-23	0.02	1.40
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	0.27	0	0.03	0.11
ITGA11	integrin, alpha 11	0.24	1.67E-13	0.32	1.37
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	2.03	5.30E-06	0.32	0.16
ITGA6	integrin, alpha 6	2.91	5.52E-10	1.46	0.52
ITGA7	integrin, alpha 7	0.38	6.46E-09	0.07	0.19
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	0.49	4.26E-06	0.20	0.43
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	0.20	3.96E-15	0.09	0.49
MCAM	melanoma cell adhesion molecule	0.33	2.43E-10	0.08	0.26
MFAP4	microfibrillar-associated protein 4	0.38	7.51E-09	0.53	1.42
MFAP5	microfibrillar associated protein 5	0.46	7.13E-07	0.38	0.86
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	4.23	0	57.95	14.07
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	7.26	1.32E-17	21.82	3.09
NFASC	neurofascin homolog (chicken)	0.38	6.14E-09	0.59	1.60
NRP1	neuropilin 1	3.10	1.14E-10	3.42	1.13
NRP2	neuropilin 2	0.24	2.23E-13	0.08	0.35
PCLKC	protocadherin LKC	0.39	1.87E-08	0.09	0.22
PKP3	plakophilin 3	0.29	1.64E-11	0.03	0.10
PLAU	plasminogen activator, urokinase	6.13	0	2.70	0.45
PODXL	podocalyxin-like	5.39	1.17E-15	4.93	0.94
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	4.33	6.67E-14	1.85	0.44
PTPRF	protein tyrosine phosphatase, receptor type, F	0.47	2.13E-06	0.09	0.19
PXN	paxillin	0.26	6.77E-13	0.04	0.15
SCARB1	scavenger receptor class B, member 1	0.45	5.37E-07	0.78	1.77
SCARF2	scavenger receptor class F, member 2	0.38	5.07E-09	0.18	0.51
SDC1	syndecan 1	2.11	2.07E-06	13.55	6.60
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1),	3.79	0	17.52	4.74
SPG7	spastic paraplegia 7 (pure and complicated autosomal recessive)	2.64	6.08E-09	2.45	0.95
THBS3	thrombospondin 3	0.47	1.88E-06	0.78	1.70
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	0.29	8.86E-12	0.08	0.27

Gene		Ratio	P-value	WNT5A	control
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	3.19	5.93E-11	16.97	5.47
TPBG	trophoblast glycoprotein	2.47	3.61E-08	5.66	2.36
U94903	Human soluble CD44 (CD44) mRNA, with exon v10 extension, partial cds. [U949	0.18	8.41E-16	0.03	0.19
VCL	vinculin	4.61	1.97E-14	12.24	2.73

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